AD_____

Award Number: DAMD17-03-1-0401

TITLE: Non-Classical NF-kappaB Forms and Bcl-3 in Breast Cancer Development and Resistance to Cancer Therapy

PRINCIPAL INVESTIGATOR: Albert S. Baldwin, Ph.D.

CONTRACTING ORGANIZATION: University of North Carolina at Chapel Hill
Chapel Hill, NC 27599

REPORT DATE: June 2006

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-06-2006	Final	19 May 2003 – 18 May 2006

4. TITLE AND SUBTITLE

Non-Classical NF-kappaB Forms and Bcl-3 in Breast Cancer Development and Resistance to Cancer Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
DAMD17-03-1-0401

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Albert S. Baldwin, Ph.D.

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of North Carolina at Chapel Hill
Chapel Hill, NC 27599

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
Breast cancer development is typified by the overexpression of growth factors and growth factor receptors, expression of cell cycle markers such as cyclin D1 and c-myc, expression of chemokines such as RANTES, and development of resistance to cancer therapies. We and others have provided evidence that the transcription factor NF-kappaB and associated activities are expressed/activated in human breast cancer. Specifically we found that the NF-kB2/p52 NF-kappaB subunit and Bcl-3 are expressed in a significant number of breast tumors. Our goals were to: (i) identify genes regulated by Her-2/neu and Bcl-3 that may be relevant to the progression of the disease, (ii) determine the mechanism whereby Bcl-3 blocks cancer-therapy induce apoptosis, and (iii) determine if Bcl-3 and the associated NF-kappaB subunit p52 are required for the development of experimental breast tumors in animal models. Our published results indicate that Bcl-3 expression suppresses p53 activation and promotes resistance to cancer therapies. Preliminary data indicate that Her-2/neu expression activates NF-kB through an Akt and IKK-dependent manner, and promotes phosphorylation of estrogen receptor (a potentially important mechanism for tamoxifen resistance and enhanced responses to estrogens).

15. SUBJECT TERMS
NF-kappaB, breast cancer therapy, suppression of apoptosis, chemoresistance, Her-2/neu

16. SECURITY CLASSIFICATION OF:				18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
					USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	32	

Table of Contents

INTRODUCTION:

Development and progression of breast cancer is characterized by dysregulation of growth factors, growth factor receptors, expression of cycle cell markers including cyclin D1 and c-myc, expression of chemokines such as RANTES, and development of resistance to cancer therapies. This type of dysregulation can be explained in a global sense through the inappropriate activation of transcription factors. We and others have provided evidence that the activity of the transcription factor NF-κB is elevated in breast cancer cell lines and in a majority of human breast tumors (for example, see Cogswell et al., 2000; Romieu-Mourez et al., 2003). Recently, Hung and colleagues provided evidence that the kinase that regulates NF-κB (IKKβ) is active in breast cancer and is involved in the inactivation of a pro-apoptotic transcription factor Foxo3a (Hu et al., 2004). Our previous analysis in human breast tumor tissues is that p52/NF-κB2 (a non-classic form of NF-κB) and Bcl-3, an IκB homologue, are upregulated in a number of breast tumors (Cogswell et al., 2000). It was also reported that Bcl-3 is upregulated in breast cancer cells following withdrawal of estrogen, leading to growth and hormone independence (Pratt et al., 2003). These findings relate to our previous publication (Westerheide et al., 2000) that Bcl-3 can directly regulate cyclin D1 gene expression (which is known to be upregulated in a majority of breast tumors). Our goals are to understand the biological roles of the NF-κB regulatory system (including Bcl-3) in breast cancer as related to development of this disease as well as in controlling cancer therapy resistance.

BODY:

The Specific Aims/Statement of Work indicate the following priorities: (year 1) identify genes and regulatory processes regulated by Bcl-3 and by Her-2/Neu, (year 2) determine mechanisms associated with the ability of Bcl-3 to alter responses to chemotherapies and radiation, and (year 3) determine if Bcl-3 and/or p52 are required for the development of experimental breast tumors using animal models.

In year one we had proposed to begin to identify genes and regulatory processes controlled by Bcl-3 and induced by Her-2/Neu. In year two, we had proposed to determine the mechanisms associated with the ability of Bcl-3 to alter responses to chemotherapy. We have made significant progress on both of these aims. We generated MCF-7 breast cancer cells that overexpress Bcl-3 and found the following: (i) these cells are highly resistant to UV-induced and chemotherapy-induced cell death and (ii) these cells exhibit a blunted p53 response (see Appendix I). Knockdown of expression of Bcl-3 restores the ability of p53 to be activated in cancer cells. In year three we have obtained the Bcl-3 null mice and are beginning crosses to determine a potential involvement of Bcl-3 (and other NF-κB subunits) in experimental breast cancer. Also in year three we have published that a variant form of IKK (IKKε) is activated in a number of cancer cell lines (including breast cancer cells) and that IKKε drives constitutive NF-κB activation through phosphorylation of the p65/RelA subunit (Adli and Baldwin, 2006).

The data on Bcl-3 suggest that breast cancer cells that express Bcl-3 will be resistant to the induction of apoptosis by cancer therapy, at least partly through the ability to suppress p53 function. Correspondingly, we analyzed Bcl-3 null cells, and found that Bcl-3 expression functions normally inhibits UV-induced and chemotherapy-induced p53 activation. This

mechanism appears to require the ability of Bcl-3 to induce Mdm2 function (see Kahsatus et al., Appendix I). Thus, the ability of Bcl-3 to suppress p53 induction appears to involve the Bcl-3 dependent activation of Mdm2 gene expression.

Additionally, although we were unable initially to maintain the Her-2/Neu-expressing H16N2 cells, we have now established those cells for subsequent experiments relating Her-2/Neu expression with Bcl-3 activity. We have initiated a microarray study to identify genes regulated by Her-2/Neu in an NF-κB-dependent manner. This array has been done once and we are currently repeating the array to confirm the findings. The initial results demonstrate an unexpected set of genes regulated (both positively and negatively) by Her-2/Neu in an NF-κB-dependent manner. These genes are likely to strongly contribute to Her-2/Neu-mediated breast cancer.

KEY RESEARCH ACCOMPLISHMENTS:

-Generation of Bcl-3 expressing MCF-7 breast cancer cell lines for studies on Bcl-3-dependent gene expression and control of chemoresistance (see Kashatus et al., Appendix I).
-Demonstration that expression of Bcl-3 strongly blocked the ability of DNA damaging stimuli (including chemotherapy) to induce cell death. The data indicate that one function of Bcl-3 expression in breast cancer is to block cancer therapy-induced cell death.
-Demonstration that the ability of p53 to be induced by chemotherapy is strongly suppressed in Bcl-3-expressing MCF-7 breast cancer cells.
-Evidence that Bcl-3 expression blocks the induction of p53-dependent genes.
-Experimental evidence that knockdown of expression of Bcl-3 leads to the ability of p53 to be induced by DNA damaging agents.
-Demonstration that the role of Bcl-3, endogenously, is to limit the activation of p53 and to limit apoptosis.
-Demonstration that expression of Bcl-3 is correlated with expression of Mdm2, the inhibitor of p53. Expression of Bcl-3 transiently in several cell types lead to the upregulation of Mdm2 gene expression.
-Obtained Bcl-3 null animals and generated primary fibroblasts that are Bcl-3 -/- for use in the analysis of the role of Bcl-3 in responses to DNA damage and apoptosis.
-Bcl-3 null cells are inhibited in their ability to induce Mdm2 gene expression, and correspondingly exhibit elevated p53 activation response.
-Obtained the compound RITA which suppresses p53 and Mdm2 interaction. Utilized this compound to show that the ability of Bcl-3 to suppress p53 activation is controlled at the level of Mdm2/p53 interaction, suggesting that the ability of Bcl-3 to upregulate Mdm2 controls its ability to block p53 activation.
-Performed an initial microarray analysis to identify NF-κB-regulated genes induced in response to expression of Her2/Neu (ErbB2). While this experiment needs to be repeated, NF-κB appears to control the expression of genes involved in promoting cell proliferation and regulating apoptosis (data not shown, until this work is repeated).
-Showed that IKKε is expressed in a variety of cancer cells, including breast cancer cells (see Adli and Baldwin, in Appendix).
-Showed that IKKε controls NF-κB activity in certain cancer cells. (Adli and Baldwin)
-Showed that Her-2/neu controls nuclear levels of NF-κB (Fig. 1, 2, 3 in Appendix).

-Showed that IKKα and IKKβ control the downstream effects of Her-2/neu (Fig. 1-3, Appendix).

REPORTABLE OUTCOMES:

We published (Kashatus et al., "Expression of the Bcl-3 Oncogene Suppresses p53 Activation", see Appendix) that Bcl-3 expression in breast tumor cells strongly suppresses cell death induced by chemotherapy. As described above, this appears to be regulated through the ability of Bcl-3 to block p53 activation through the upregulation of the p53 inhibitor Hdm2/mdm2. This is a key finding regarding the function of Bcl-3 and relevant to understanding why breast cancer may be resistant to cancer therapy treatment.

We published (Adli and Baldwin, 2006, see Appendix) that IKKε is upregulated in several breast cancer cell lines and controls NF-κB in those cells through phosphorylation of the p65 NF-kB subunit.

CONCLUSIONS:

Bcl-3, which is described as an oncoprotein in certain leukemias and lymphomas, is expressed in breast cancer animal models and in human breast tumors. Our present data indicate the very interesting possibility that Bcl-3 controls p53 tumor suppressor function in a negative manner while functioning to promote cell proliferation through upregulation of Cyclin D1 (often overexpressed in breast cancer). The data would suggest that breast tumors with Bcl-3 expression are actually inhibited for the tumor suppressor function of p53 via the potential upregulation of Mdm2. The data may also explain why Bcl-3-expressing tumors are chemo- or radioresistant and raise the potential of inhibiting Bcl-3 as a new therapeutic direction. The data also raise the interesting possibility that Mdm2 has functions separate from inhibiting p53 and that Bcl-3 promotes this process.

IKKε is indentified as a mediator of NF-κB function in cancer cells, including breast cancer cells.

Her-2/neu is shown to induce NF-κB nuclear accumulation in a manner dependent on Akt, and on both IKKα and IKKβ.

REFERENCES:

Adli, M. and A. Baldwin. 2006. IKKi/ε controls constitutive, cancer cell-associated NF-κB activity via regulation of ser-536 p65/RelA phosphorylation. J. Biol. Chem. 281, 26976-26984.

Cogswell, P.C., D. Guttridge, W. Funkhouser, and A. Baldwin. 2000. Selective activation of NF-κB subunits in human breast cancer: potential roles for NF-κB2/p52 and Bcl-3. Oncogene 19, 1123-1131.

Hu, M.C., D.F. Lee, W. Xia, L.Golfman, F. Yang, J. Yang, Y. Zou, S. Bao, N. Hanada, H. Saso, R. Kobayashi, and M.C. Hung. 2004. IKK promotes tumorigenesis through inhibition of Forkhead Foxo3a. Cell 117, 225-237.

Kashatus, D., P. Cogswell, and A. Baldwin. 2006. Expression of the Bcl-3 proto-oncogene suppresses p53 activation. Genes and Dev. 20, 225-235.

Pratt, M.A., T. Bishop, D. White, G. Yasvinski, M. Menard, M. Niu, and R. Clarke. 2003. Estrogen withdrawal-induced NF-κB activity and Bcl-3 expression in breast cancer cells: roles in growth and hormone independence. Mol. Cell. Biol. 23, 6887-6900.

Romieu-Mourez, R., D. Kim, S.Shin, E. Demico, D. Landesman-Bollag, D. Seldin, R. Cardiff, and G. Sonenshein. 2003. Mouse mammary tumor virus c-rel transgenic mice develop breast tumors. Mol. Cell. Biol. 23, 5738-5754.

Westerheide, S., M. Mayo, V. Anest, J. Hanson, and A. Baldwin. 2001. The putative oncoprotein Bcl-3 induces cyclin D1 to induce G1 transition. Mol. Cell. Biol. 21, 8428-8436.

APPENDIX

1. Kashatus et. al
2. Adli and Baldwin
3. Her-2 Figures (1-3)

Expression of the Bcl-3 proto-oncogene suppresses p53 activation

David Kashatus, Patricia Cogswell and Albert S. Baldwin

Genes & Dev. 2006 20: 225-235; originally published online Dec 29, 2005;
Access the most recent version at doi:10.1101/gad.1352206

Expression of the Bcl-3 proto-oncogene suppresses p53 activation

David Kashatus,[1,2] Patricia Cogswell,[2] and Albert S. Baldwin[1,2,3,4]

[1]Curriculum in Genetics and Molecular Biology, [2]Lineberger Comprehensive Cancer Center, and [3]Department of Biology, University of North Carolina School of Medicine, Chapel Hill, North Carolina 27599, USA

While Bcl-3 expression in cancer was originally thought to be limited to B-cell lymphomas with a 14;19 chromosomal translocation, more recent evidence indicates that expression of this presumptive oncoprotein is significantly more widespread in cancer. However, an oncogenic role for Bcl-3 has not been clearly identified. Experiments presented here indicate that Bcl-3 is inducible by DNA damage and is required for the induction of Hdm2 gene expression and the suppression of persistent p53 activity. Furthermore, constitutive expression of Bcl-3 suppresses DNA damage-induced p53 activation and inhibits p53-induced apoptosis through a mechanism that is at least partly dependent on the up-regulation of Hdm2. The results provide insight into a mechanism whereby altered expression of Bcl-3 leads to tumorigenic potential. Since Bcl-3 is required for germinal center formation, these results suggest functional similarities with the unrelated Bcl-6 oncoprotein in suppressing potential p53-dependent cell cycle arrest and apoptosis in response to somatic hypermutation and class switch recombination.

[*Keywords*: Apoptosis; Bcl-3; Hdm2; NF-κB; p53]

Supplemental material is available at http://www.genesdev.org.

Received July 5, 2005; revised version accepted November 17, 2005.

Bcl-3 was first identified through cloning of the t(14;19) breakpoint junction, which occurs in a subset of B-cell chronic lymphocytic leukemias (B-CLLs) (McKeithan et al. 1987). A member of the ankyrin-repeat-containing IκB family of NF-κB inhibitors (Hayden and Ghosh 2004), Bcl-3 is apparently unique in that, unlike other IκBs, its localization is nuclear and it contains a transactivation domain. Studies have shown that Bcl-3 preferentially binds to NF-κB p50 and p52 homodimers (Bours et al. 1993; Nolan et al. 1993) and, through its interaction with coactivators such as CBP/p300, SRC-1, and Tip60 (Na et al. 1998; Dechend et al. 1999) and corepressors such as HDAC1 (Wessells et al. 2004), can both activate and repress transcription of target genes. Importantly, genetic knockout of Bcl-3 leads to impaired microarchitecture in spleen and lymph nodes with associated germinal center defects (Franzoso et al. 1997; Schwarz et al. 1997).

Relatively high levels of Bcl-3 expression in certain B-CLLs suggests that it may play a direct role in oncogenesis. In this regard, expression of Bcl-3 is sufficient to transform NIH3T3 cells (Viatour et al. 2004). Importantly, Bcl-3 has now been shown to be more widely expressed in cancer, with expression in a significant number of breast cancers (Cogswell et al. 2000), nasopharyngeal carcinomas (Thornburg et al. 2003), lymphomas (Canoz et al. 2004), and hepatocellular carcinomas and pancreatic cancers (B. O'Neil, unpubl.). The findings that Bcl-3, in complex with p52, can promote transcription of the genes encoding the cell cycle regulator cyclin D1 and the anti-apoptotic Bcl-2 protein suggest one potential oncogenic mechanism (Westerheide et al. 2001; Viatour et al. 2003), but a more complete understanding of the role of Bcl-3 in human cancers is still lacking.

p53 is a crucial guardian of genomic integrity, and its importance as a tumor suppressor is underscored by the fact that it is either mutated or otherwise dysregulated in the majority of human cancers (Vousden and Lu 2002; Vousden and Prives 2005). Normally, p53 remains at low levels through its interaction with the E3 ubiquitin ligase, Hdm2. Upon cellular stress or oncogene activation, this interaction is disrupted and p53 rapidly accumulates in the nucleus, where it can activate a number of target genes, including the cyclin-dependent kinase inhibitor p21 (el-Deiry et al. 1993), pro-apoptotic genes Noxa and Puma (Oda et al. 2000; Nakano and Vousden 2001; Yu et al. 2001), and its own inhibitor Hdm2 (Barak et al. 1993). Recent evidence also suggests that p53 can play a direct apoptotic role in the mitochondria, independent of its transcriptional function (Chipuk et al. 2004). Ultimately, depending on the type and intensity of the signal, as well as the cellular context, p53 stabilization results in either cell cycle arrest or apoptosis. Loss of p53 function, through either mutation or an alternative

[4]Corresponding author.
E-MAIL abaldwin@med.unc.edu; FAX (919) 966-0444.
Article published online ahead of print. Article and publication date are at http://www.genesdev.org/cgi/doi/10.1101/gad.1352206.

mechanism such as overexpression of Hdm2, leads to dysregulated growth, protection against apoptosis, and genomic instability, allowing for the accumulation of secondary mutations (Bond et al. 2005; Vousden and Prives 2005).

Based on the ability of NF-κB to regulate cell death pathways (Ghosh and Karin 2002; Kucharczak et al. 2003) and on recent studies that suggest a link between Bcl-3 and the DNA damage response (Boulton et al. 2002; Watanabe et al. 2003), we have investigated a potential role for Bcl-3 in regulating the apoptosis response to DNA damage. We find that Bcl-3 is transiently up-regulated by DNA damage and that constitutive expression of Bcl-3 in MCF-7 cells leads to a strong suppression of DNA damage-induced cell death without a significant effect on NF-κB-regulated anti-apoptotic genes. Importantly, Bcl-3 expression leads to suppression of p53 induction following DNA damage, resulting in the inhibition of expression of the p53 target genes Noxa and Puma. Analysis of Bcl-3-null mouse embryonic fibroblasts (MEFs) or cancer cells knocked down for Bcl-3 reveals that loss of Bcl-3 leads to an enhanced p53 response. One mechanism by which this inhibition occurs is through Bcl-3-mediated induction of the p53 inhibitor Hdm2. Both stable and transient overexpression of Bcl-3 leads to increased Hdm2 expression, and small interfering RNA (siRNA)-mediated knockdown of Bcl-3 blocks expression of Hdm2. Analysis of Bcl-3-null fibroblasts reveals that Bcl-3 is required for the induction of Mdm2 gene expression and for limiting the p53 activation response. Disruption of the p53–Hdm2 interaction in Bcl-3-expressing cells functions to rescue the p53 inhibition and apoptotic response. Furthermore, the data support a model whereby Bcl-3, like the unrelated oncoprotein Bcl-6, functions to suppress p53-dependent cell cycle arrest and apoptosis in germinal center B cells undergoing somatic mutation and class switch recombination. Our results provide significant new insights into oncogenic mechanisms associated with Bcl-3 and provide evidence that Bcl-3 is required for p53 to induce expression of Hdm2 gene expression.

Results

Elevated Bcl-3 expression inhibits DNA damage-induced apoptosis

In addition to its relatively high expression in B-CLLs carrying the t(14;19) translocation, Bcl-3 has also been found to be elevated in a number of other human cancers, including nasopharyngeal carcinoma (Thornburg et al. 2003), breast cancer (Cogswell et al. 2000), lymphoma (Canoz et al. 2004), and hepatocellular carcinoma (B. O'Neil, unpubl.). To understand the consequences of Bcl-3 constitutive expression and to investigate its potential role in driving oncogenesis, we engineered the MCF-7 breast cancer cell line to express relatively high levels of Bcl-3 (MCF-7B). Expression of Bcl-3 in the MCF-7B cells is comparable to its expression in several cancer cell lines, including Karpas 299 cells, which have been previously reported to express high levels of Bcl-3 (Nishikori et al. 2003), and the fibrosarcoma line, HT-1080 (Fig. 1A).

One characteristic shared by many human cancers is the inability to appropriately respond to genomic insults. To determine if Bcl-3 overexpression might contribute to this deficiency, we treated MCF-7 and MCF-7B cells with either UV-C or the DNA damage-inducing drug cisplatin and measured the apoptotic response. Intriguingly, MCF-7B cells exhibit a significant resistance to DNA damage-induced cell death (Fig. 1B). As expected, the DNA damage-induced cell death in the parental MCF-7 line is dependent on p53 activity, as cotreatment with the p53 inhibitor pifithrin-α (Komarov et al. 1999) inhibits the apoptotic response following UV treatment (Fig. 1C).

Notably, the protective effects of Bcl-3 do not extend to non-p53-dependent inducers of apoptosis, as treatment with the drug staurosporine results in similar levels of apoptosis in both MCF-7 and MCF-7B cells (Supplementary Fig. 1A). Somewhat surprisingly, no increase was observed in the MCF-7B cells of expression of

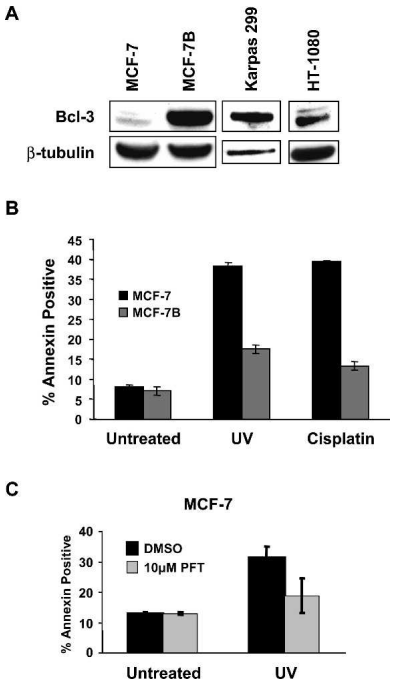


Figure 1. Overexpression of Bcl-3 inhibits DNA damage-induced apoptosis. (*A*) Expression of Bcl-3 in MCF-7, MCF-7B, Karpas 299, and HT-1080 cells. Western blots of extracts from indicated cell lines were probed with antibodies against Bcl-3 and β-tubulin. (*B*) MCF-7B cells are protected against DNA damage-induced apoptosis. MCF-7 and MCF-7B cells were left untreated or were treated with 40 J/m^2 UV-C or 10 μg/mL cisplatin as indicated. Eighteen hours after treatment, apoptosis was measured by flow cytometric analysis of Annexin-V staining. (*C*) UV-induced apoptosis in MCF-7 cells is p53 dependent. MCF-7 cells were treated with DMSO, 10 μM pifithrin-α, 40 J/m^2 UV-C plus DMSO, or 40 J/m^2 UV-C plus 10 μM pifithrin-α as indicated. Eighteen hours after treatment, apoptosis was measured by flow cytometric analysis of Annexin-V staining.

known anti-apoptotic genes that are reportedly regulated by NF-κB following treatment with UV or cisplatin (D. Kashatus, unpubl.). As such, we sought to investigate other potential mechanisms for this anti-apoptotic activity.

Bcl-3 inhibits the p53 response to DNA damage

As p53 is a critical mediator of the apoptotic response following DNA damage (Vousden and Prives 2005), and given that DNA damage-induced apoptosis in MCF-7 cells is p53 dependent (Fig. 1C), we sought to investigate whether expression or activation of this tumor suppressor is affected by constitutive expression of Bcl-3. Interestingly, Bcl-3 protein levels increase transiently between 2 and 6 h following UV treatment in the parental MCF-7 cells, returning to basal levels by 24 h, suggesting that this oncoprotein may have a role in the UV response. The increase in Bcl-3 protein is likely to be at the level of protein stability as we detect no increase in Bcl-3 mRNA following UV treatment (D. Kashatus, unpubl.). The transient nature of the response is consistent with the report that p53 can negatively regulate Bcl-3 protein (Rocha et al. 2003), as the loss of Bcl-3 coincides with the accumulation of p53.

As expected, p53 is induced in the parental MCF-7 cells in response to UV treatment, with peak levels reached ~8 h following treatment. Intriguingly, the p53 response in MCF-7B cells is significantly muted, with peak p53 protein levels being considerably lower than in the parental cells (Fig. 2A). This down-regulation of the p53 response is not correlated with reduced p53 mRNA levels (Fig. 2B). To ensure that the resulting suppression of the p53 response by Bcl-3 is not a consequence of creating and selecting stable cells, we transiently expressed Flag-tagged Bcl-3 in parental MCF-7 cells and treated the cells with UV. Consistent with the results from the stable lines, increased expression of Bcl-3 leads to a decrease in the levels of p53 protein 4 h following UV treatment (Fig. 2C). Further, we transfected expression vectors for p53 plus Bcl-3 or p53 plus empty vector along with the pg13 p53 responsive reporter (el-Deiry et al. 1993) into parental MCF-7 cells to test p53 transcriptional activity in the presence of excess Bcl-3. As expected, p53 activated the luciferase reporter while coexpression of Bcl-3 partially suppressed this response (Fig. 2D). Basal p53 activity is very low in these cells, thus any decrease in basal p53 activity upon Bcl-3 overexpression falls below the detection limit of the assay. Taken together, these results indicate that expression of Bcl-3 can suppress the activation of p53 (see Discussion).

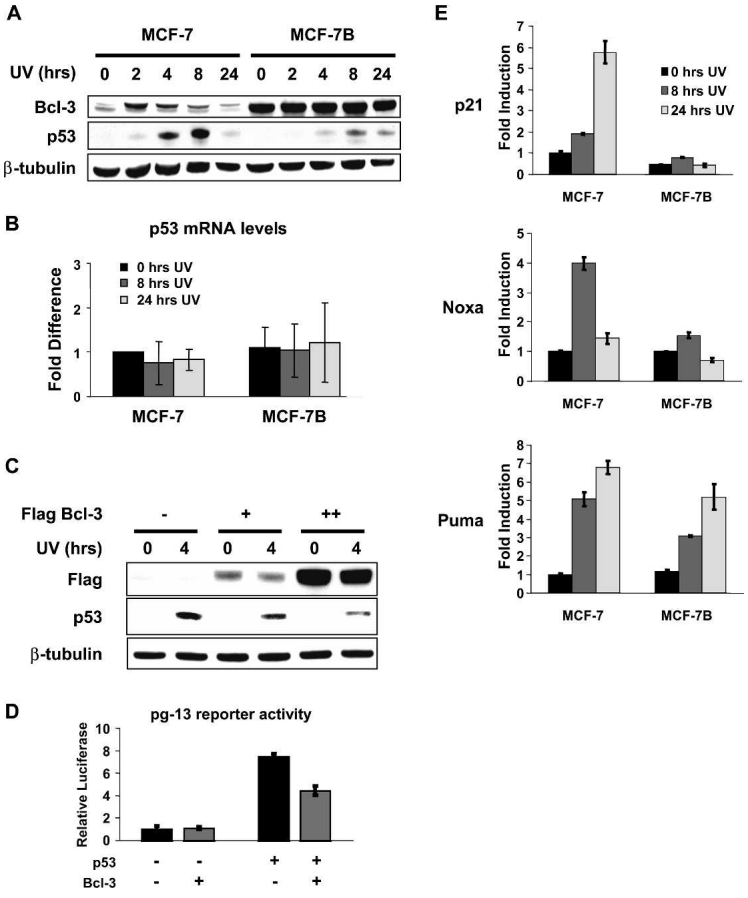


Figure 2. Bcl-3 overexpression inhibits DNA damage-induced p53 activity. (*A*) UV-induced p53 protein levels are reduced in MCF-7B cells. MCF-7 and MCF-7B cells were treated with 40 J/m^2 UV-C for the indicated times, and Western analysis was performed on whole-cell extracts using antibodies against Bcl-3, p53, and β-tubulin. (*B*) Overexpression of Bcl-3 does not affect p53 mRNA levels. MCF-7 and MCF-7B cells were treated with 40 J/m^2 UV-C for the indicated times, and relative expression of p53 was measured by quantitative real-time PCR. (Lane *1*) Expression levels were normalized to expression of glucuronidase-β, and the values represent the fold increase or decrease relative to untreated MCF-7 cells. (*C*) Transient expression of Bcl-3 leads to decreased p53 protein levels following UV treatment. MCF-7 cells were transfected with either empty vector or 2 or 4 μg of pCMV–Flag-Bcl-3. Two days after transfection, the cells were left untreated or were treated with 50 J/m^2 UV-C for 4 h, and Western analysis was performed with antibodies against the Flag epitope, p53, or β-tubulin. (*D*) Transient expression of Bcl-3 inhibits p53 transcriptional activity. MCF-7 cells were transfected with 50 ng of pg-13–luciferase and 5 ng of renilla luciferase plus 100 ng of pCMV–Flag-Bcl-3 and pCMV–Flag-p53 where indicated. Firefly luciferase activity was measured and normalized to renilla luciferase. (Lane *1*) Values represent fold increase over basal activity. (*E*) DNA damage-induced expression of p53 target genes is lost in MCF-7B cells. MCF-7 and MCF-7B cells were treated with 40 J/m^2 UV-C for the indicated times, and relative expression of p21, Noxa, and Puma was measured by quantitative real-time PCR. (Lane *1*) Expression levels were normalized to expression of glucuronidase-β, and the values represent the fold increase or decrease relative to untreated MCF-7 cells.

To determine the effect of Bcl-3 expression on the induction of p53 target genes, real-time quantitative PCR was performed on cDNA generated from MCF-7 and MCF-7B cells exposed to UV. The cyclin-dependent kinase inhibitor p21 is an important target of p53 that functions in p53-dependent cell cycle arrest. Real-time PCR analysis of p21 mRNA indicates that Bcl-3 expression leads to an almost complete loss of p21 induction by UV (Fig. 2E). Other important gene targets of p53 encode pro-apoptotic proteins, including Noxa and Puma, members of the BH3-only class of Bcl-2 homologs. Recent knockouts of these p53 targets indicate that they are essential effectors of p53-dependent apoptosis (Jeffers et al. 2003; Villunger et al. 2003). Analysis of these gene products indicates that, as with p21, constitutive Bcl-3 expression leads to a loss of their induction following UV treatment (Fig. 2E). Notably, while p21 and Noxa expression are almost completely abrogated, the levels of Puma are only modestly inhibited, consistent with reports of p53-dependent and p53-independent induction of this gene (Jeffers et al. 2003).

Knockdown or knockout of Bcl-3 expression leads to enhanced p53 activation following DNA damage

To further analyze the role of Bcl-3 in DNA damage-induced p53 function, we analyzed the outcome of knockdown of Bcl-3 in HT1080 fibrosarcoma cells since these cells express a functional form of p53 (Suzuki et al. 2003) as well as elevated Bcl-3. siRNA against Bcl-3, but not the control siRNA, leads to a near complete loss of the faster migrating form of Bcl-3 and to a significant reduction in the slower migrating form. Importantly, knockdown of expression of Bcl-3 generates enhanced basal and UV-induced p53 expression. These results indicate that elevated expression of Bcl-3, at least in certain p53 wild-type cancer cells, represses the activation potential of p53. To extend these studies and to analyze the outcome of loss of Bcl-3 through a mechanism independent of siRNA knockdown, we analyzed the p53 response in Bcl-$3^{+/+}$ and Bcl-$3^{-/-}$ primary MEFs. Exposure of these cells to UV leads to the expected p53 response in wild-type cells, but to elevated levels of p53 at the 24-h time point in Bcl-3-null cells (Fig. 3B). Consistent with the observed p53 response in Bcl-3-null fibroblasts, treatment of these cells with UV leads to an increase in apoptosis compared with the wild-type cells (Fig. 3C). Importantly, cotreatment with pifithrin-α leads to a reduction in the apoptotic response to UV in the Bcl-$3^{-/-}$ cells, indicating that this response is at least partly dependent on p53 activity (Fig. 3C). In addition, loss of Bcl-3 does not sensitize MEFs to TNFα-induced apoptosis (Supplementary Fig. 1B) consistent with the idea that the protection it provides is specific for p53-dependent inducers of apoptosis. These results are consistent with a role for Bcl-3 in suppressing the p53 activation response, both when Bcl-3 is constitutively expressed at high levels and when Bcl-3 is transiently induced in response to DNA damage.

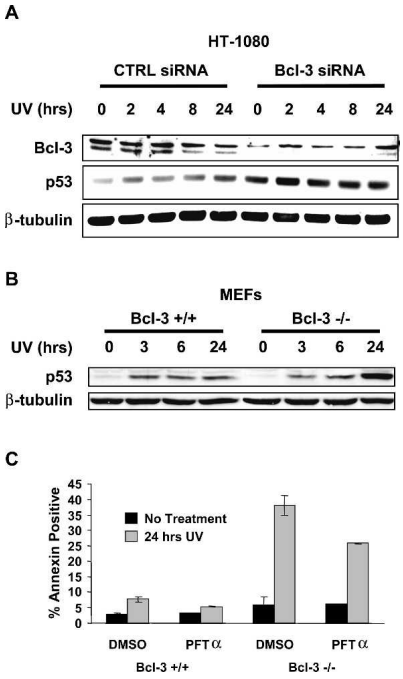


Figure 3. Loss of Bcl-3 leads to increased levels of p53 following DNA damage and sensitivity to DNA damage-induced apoptosis. (*A*) Knockdown of Bcl-3 results in an increase of UV-induced p53 protein. HT-1080 cells were transfected with a control siRNA or an siRNA targeting Bcl-3. Forty-eight hours after transfection, cells were treated with 40 J/m^2 UV-C for the indicated time points. Western analysis was performed on whole-cell extracts using antibodies against Bcl-3, p53, and β-tubulin. (*B*) Bcl-3-null MEFs have increased p53 levels following UV treatment. Wild-type and Bcl-3-deficient MEFs were treated with 40 J/m^2 UV-C for the indicated time points. Western analysis was performed on whole-cell extracts using antibodies against p53 and β-tubulin. (*C*) Bcl-3-null MEFs are sensitized to p53-dependent UV-induced apoptosis. Bcl-$3^{+/+}$ and Bcl-$3^{-/-}$ MEFs were treated with DMSO, 10 μM pifithrin-α, 40 J/m^2 UV-C plus DMSO, or 40 J/m^2 UV-C plus 10 μM pifithrin-α as indicated. Eighteen hours after treatment, apoptosis was measured by flow cytometric analysis of Annexin-V staining.

Bcl-3 expression leads to an increase in Hdm2 levels

p53 activation is controlled through modifications (such as phosphorylation) and through interactions with its inhibitor Hdm2 (Vousden and Prives 2005). Analysis of UV-induced phosphorylation of p53 (Ser 15 and Ser 20) in MCF-7B cells indicated that this modification tracked with proteins levels (D. Kashatus, unpubl.), suggesting that Bcl-3 expression does not block p53 Ser 15 or Ser 20 phosphorylation. To analyze how high levels of expression of Bcl-3 might lead to the loss of p53 induction by DNA damage, we investigated the levels of the p53 inhibitor Hdm2. Western analysis of MCF-7 and MCF-7B cells reveals that MCF-7B cells exhibit consistently higher Hdm2 levels (Fig. 4A, first panel), providing a potential mechanism for the inhibition of p53 seen in these cells. Importantly, transient overexpression of Bcl-3 in parental MCF-7 cells also leads to increased Hdm2 protein (Fig. 4A, second panel), confirming that the increase

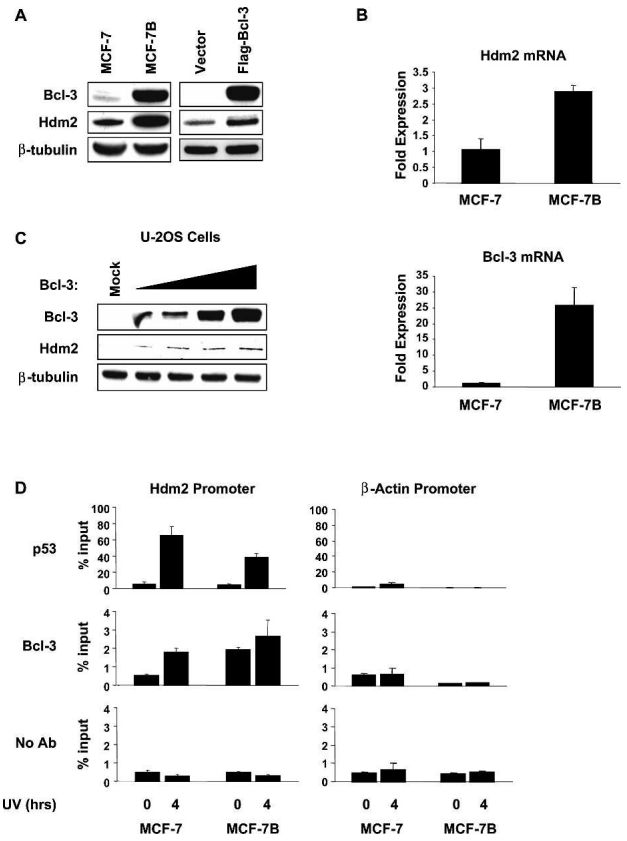


Figure 4. Overexpression of Bcl-3 leads to an increase in Hdm2 expression. (*A*) Both transient and stable overexpression of Bcl-3 lead to higher basal levels of Hdm2 protein. Whole-cell extracts were prepared from MCF-7 and MCF-7B cells as well as MCF-7 cells transfected with 8 μg of either empty vector or pCMV–Flag-Bcl-3, and Western analysis was performed using antibodies against Bcl-3, Hdm2, and β-tubulin. (*B*) MCF-7B cells have higher basal levels of Hdm2 RNA. Real-time quantitative PCR was performed on cDNA prepared from MCF-7 and MCF-7B cells using primers specific for Bcl-3 and Hdm2. (Lane *1*) Expression levels were normalized to expression of glucuronidase-β, and values represent fold difference relative to MCF-7 for each gene tested. (*C*) Transient overexpression of Bcl-3 leads to an increase in Hdm2 levels in U-2OS cells. U-2OS cells were transfected with 0–200 ng of pCMV–Flag-Bcl-3 in 50-ng increments. Total DNA content was brought to 200 ng with pCMV–Flag vector. Forty-eight hours after transfection, Western analysis was performed on whole-cell extracts using antibodies against Bcl-3, Hdm2, and β-tubulin. (*D*) Bcl-3 is present at the Hdm2 promoter at higher levels in MCF-7B cells. MCF-7 and MCF-7B cells were treated with 40 J/m^2 UV-C for either 0 or 4 h, and ChIP was performed using antibodies specific for p53 or Bcl-3, or no antibody. Real-time quantitative PCR was performed on precipitated DNA using primers specific for the p2 promoter region of the Hdm2 gene and for the promoter region of the β-actin gene. Values are normalized against the input DNA and are represented as the percentage of input for each given sample. Each value represents the mean of three independent measurements of the precipitated DNA, and the error bars represent one standard deviation. The experiment was repeated three times with identical results.

seen in the stable cells is not an artifact of clonal selection. This increase in Hdm2 in MCF-7B cells is also evident at the RNA level, as real-time quantitative PCR indicates a roughly threefold increase in Hdm2 levels in MCF-7B cells relative to parental MCF-7 cells. To show that this effect is not unique to MCF-7 cells, we transiently transfected Bcl-3 into the U-2OS osteosarcoma line. Consistent with the results from MCF-7 cells, increasing levels of Bcl-3 leads to proportionately increased levels of Hdm2 protein in these cells (Fig. 4C).

To determine whether the Bcl-3-dependent increase in Hdm2 might be due to a direct role for Bcl-3 at the Hdm2 promoter, we performed chromatin immunoprecipitation (ChIP) analysis using PCR primers specific for the P2 promoter region of the Hdm2 promoter, flanking the two conserved p53-binding sites. As reported for other p53 inducible genes (Espinosa et al. 2003), there are relatively low levels of p53 at the promoter under basal conditions, with a severalfold increase following UV treatment (Fig. 4D). Notably, the levels of p53 at the promoter 4 h post-UV are lower in the MCF-7B cells when compared with the parental MCF-7 cells, consistent with the lower levels of stabilized p53 in these cells. Intriguingly, Bcl-3 is also present at the Hdm2 promoter at levels consistent with its expression in the cells. In MCF-7 cells, Bcl-3 is not detectable basally but increases as the protein levels increase, while in the MCF-7B cells it is present both basally and after UV treatment (Fig. 4D). As a control, it is shown that neither Bcl-3 nor p53 is present at significant levels at the β-actin promoter.

The observed recruitment of Bcl-3 to the Hdm2 promoter led us to investigate potential binding sites for Bcl-3 in the Hdm2 P2 promoter region. We identified a putative NF-κB-binding site 275 base pairs (bp) upstream of the exon 2 start site that is conserved in the mouse Mdm2 promoter (Supplementary Fig. 2A). Intriguingly, gel shift analysis using a ^{32}P-labeled oligonucleotide probe specific for this site shows increased binding to the probe in the MCF-7B cells compared with the parental MCF-7 cells. Further analysis using antibodies specific for various NF-κB subunits shows that the complex binding this site contains both p50 and p52, known binding partners of Bcl-3 (Supplementary Fig. 2B).

In addition, we performed reporter assays using Hdm2 P2 luciferase reporters containing various portions of the P2 promoter. The two reporters containing the region of the promoter that includes the NF-κB-binding site (Hdm2–Luc-01 and Hdm2–Luc-02) consistently exhibit increased activity in the MCF-7B cells compared with the parental MCF-7 cells. Conversely, the reporter construct that lacks the region containing the NF-κB site (Hdm2–Luc-03) exhibits no difference between the two cell types (Supplementary Fig. 2C). While we cannot rule out the existence of other important regulatory sites in this deleted region, these data are consistent with the importance of this binding site for Bcl-3-induced Hdm2 transcription.

Bcl-3 is required for Hdm2 gene expression

Given that Bcl-3 is recruited to the Hdm2 promoter following UV treatment (Fig. 4D), and that its elevated expression leads to increased levels of Hdm2, we sought to determine whether Bcl-3 is required for Hdm2 expression. Knockdown of Bcl-3 by RNA interference (RNAi) in both MCF-7 and Karpas 299 cells leads to a near complete loss of Hdm2 expression, consistent with Bcl-3 being required for basal Hdm2 expression in these cells (Fig. 5A). Note that Karpas 299 cells are reported to express mutant p53 (Hubinger et al. 2001), suggesting a lack of involvement of p53 in control of Hdm2 gene expression in these cells. To further test our hypothesis, primary fibroblasts from mice lacking Bcl-3 and their wild-type littermates were analyzed following DNA damage. When wild-type or Bcl-3-null MEFs are treated with either UV or the DNA damage-inducing drug cisplatin, cells lacking Bcl-3 fail to induce mdm2 mRNA to the levels seen in wild-type cells (Fig. 5B). Consistent with this, the induction of Mdm2 protein levels is severely repressed in Bcl-$3^{-/-}$ cells as compared with their wild-type counterparts (Fig. 5C). To determine if suppression of Bcl-3 inhibits the induction of Hdm2 in another cell type, siRNA was utilized to suppress Bcl-3 expression in HT-1080 cells, which express functional p53 (Suzuki et al. 2003). Knockdown of Bcl-3 in HT-1080 cells also leads to a suppression of the induction of Hdm2 following UV treatment (Fig. 5D). These results demonstrate an important role for Bcl-3 in controlling Hdm2/mdm2 gene expression in several cell types.

Disruption of the p53–Hdm2 interaction in MCF-7B cells rescues the DNA damage-induced p53 response

To determine if Bcl-3-mediated repression of DNA damage-induced p53 stability involves Hdm2, we analyzed whether disruption of the p53–Hdm2 interaction is able to rescue p53 responsiveness in MCF-7B cells. The recently described compound RITA (reactivation of p53 and induction of tumor cell apoptosis) has been shown to bind to p53 both in vitro and in vivo and to prevent its interaction with Hdm2 (Issaeva et al. 2004). MCF-7 and MCF-7B (Bcl-3-expressing) cells were exposed to RITA or to diluent, and to UV treatment. The results demonstrate the expected p53 induction in MCF-7 cells and the strong suppression of p53 activation in MCF-7B cells. Importantly, exposure of the MCF-7B cells to RITA leads to a rescue of the Bcl-3-controlled repression of p53 as evidenced by higher levels of p53 stabilization than in cells treated with UV and vehicle control (Fig. 6A). To test the Hdm2 dependence in another way, we transfected MCF-7B cells with either a control siRNA or an siRNA specific for Hdm2. Consistent with the response following RITA treatment, knockdown of Hdm2 in MCF-7B cells leads to a rescue of the p53 response following UV treatment (Fig. 6B). Collectively, these data indicate that the repression of p53 induction seen in MCF-7B cells is dependent on the p53–Hdm2 interaction, consistent with the model that increased Hdm2 levels in MCF-7B cells are responsible for the repression.

To determine if the protection against apoptosis observed in MCF-7B cells (Fig. 1) is dependent on Hdm2-mediated repression of p53, MCF-7 and MCF-7B cells

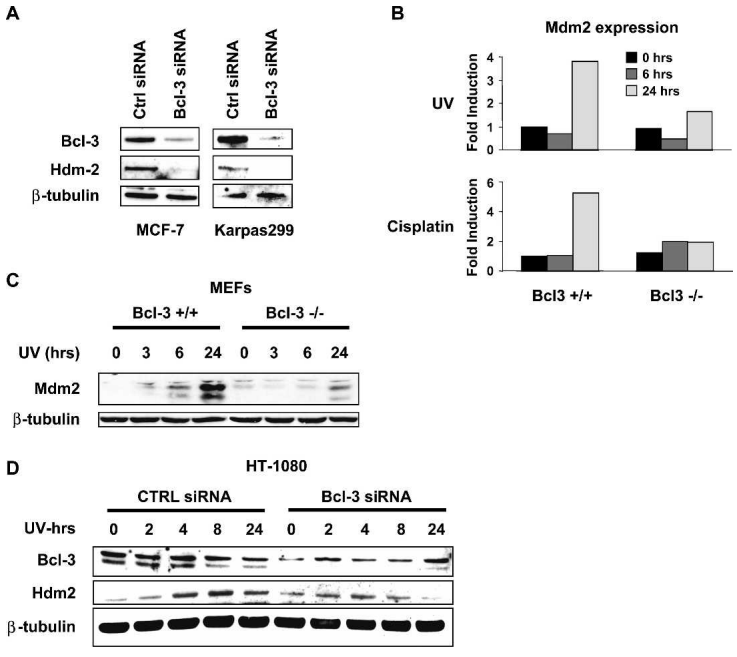


Figure 5. Loss of Bcl-3 leads to a decrease in basal and DNA damage-inducible Hdm2 expression. (*A*) Knockdown of Bcl-3 in human cancer cells leads to loss of Hdm2 expression. MCF-7 and Karpas 299 cells were transfected with either control siRNAs or siRNAs specific for Bcl-3. Forty-eight hours after transfection, Western analysis was performed on whole-cell extracts using antibodies specific for Bcl-3, Hdm2, and β-tubulin. (*B*) DNA damage fails to induce Mdm2 RNA in Bcl-3-deficient fibroblasts. Wild-type and Bcl-3-null MEFs were treated with either 40 J/m^2 UV-C or 10 μg/mL cisplatin for the indicated times, and Mdm2 gene expression was measured by quantitative real-time PCR. (Lane *1*) Expression levels were normalized to expression of GAPDH, and the values represent the fold increase or decrease relative to untreated wild-type MEFs. (*C*) DNA damage fails to induce Mdm2 protein in Bcl-3-deficient fibroblasts. Wild-type and Bcl-3-null MEFs were treated with 40 J/m^2 UV-C for the indicated times, and Western blots were performed on whole-cell extracts using antibodies specific for Mdm2 and β-tubulin. (*D*) Knockdown of Bcl-3 impairs the ability of DNA damage to induce Hdm2 in HT-1080 cells. HT-1080 cells were transfected with either control siRNAs or siRNAs specific for Bcl-3. Forty-eight hours after transfection, cells were treated with 40 J/m^2 UV-C for the indicated times, and Western analysis was performed on whole-cell extracts using antibodies specific for Bcl-3, Hdm2, and β-tubulin.

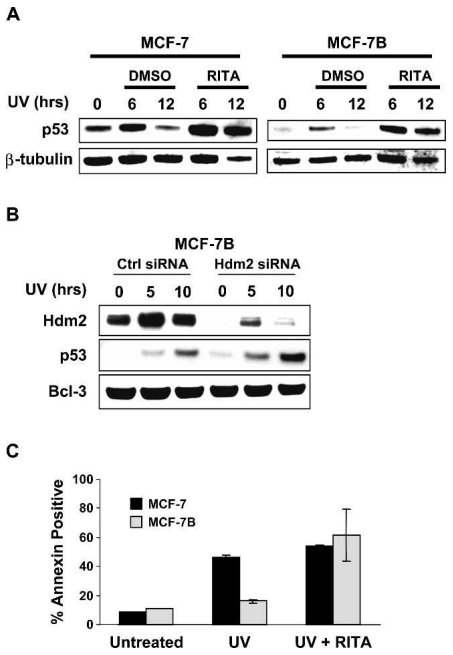


Figure 6. Disruption of the p53–Hdm2 interaction rescues the effects of Bcl-3 overexpression. (*A*) Disruption of the p53–Hdm2 interaction restores the ability of UV to induce p53 in MCF-7B cells. MCF-7 and MCF-7B cells were pretreated for 30 min with either DMSO or 2 μM RITA and then treated with 40 J/m^2 UV-C for the indicated times. Whole-cell extracts were prepared and subjected to Western blot analysis using antibodies specific for p53 and β-tubulin. (*B*) Knockdown of Hdm2 restores the ability of UV to induce p53 in MCF-7B cells. MCF-7B cells were transfected with either a control siRNA or an siRNA specific for Hdm2. Forty-eight hours after transfection, cells were treated with 40 J/m^2 UV-C for the indicated times. Whole-cell extracts were prepared and subjected to Western blot analysis using antibodies specific for p53, Hdm2, and β-tubulin. (*C*) Disruption of the p53–Hdm2 interaction restores the ability of UV to induce apoptosis in MCF-7B cells. MCF-7 and MCF-7B cells were left untreated, or were treated with 40 J/m^2 UV-C or with 40 J/m^2 UV-C plus 2 μM RITA. Eighteen hours after treatment, apoptosis was measured by flow cytometric analysis of Annexin-V staining.

were either treated with UV alone or with UV plus RITA and the apoptotic response was measured. Consistent with the data from Figure 1, MCF-7B cells are significantly protected against apoptosis when treated with UV alone. However, cotreatment with RITA rescues the apoptotic defect in MCF-7B cells. Importantly, cotreatment with UV and RITA results in equivalent levels of apoptosis in MCF-7 and MCF-7B cells, demonstrating that the interaction between p53 and Hdm2 is critical for the observed apoptotic differences in the two cell lines (Fig. 6C). Similarly, siRNA-mediated knockdown of Hdm2 is able to restore the UV-induced apoptotic response in MCF-7B cells (Supplementary Fig. 3), albeit to a lesser extent than treatment with RITA. This is likely due to a <100% transfection efficiency in these cells leading to an incomplete knockdown of Hdm2. These data further demonstrate the importance of the p53–Hdm2 interaction on the antiapoptotic effects of Bcl-3. Collectively, these data indicate that the Bcl-3-mediated protection against DNA damage-induced apoptosis involves the regulated interaction between p53 and Hdm2 and supports the hypothesis that Bcl-3-controlled induction of Hdm2 is responsible for the observed repression of p53.

Discussion

The data presented here reveal a surprising pro-oncogenic role for the oncoprotein Bcl-3, namely, its ability to suppress the activation of the p53 tumor suppressor protein in response to DNA damage. A major proposed mechanism in this regulatory circuit is the ability of Bcl-3 to induce the expression of Hdm2, the inhibitor of p53. The experiments demonstrate that expression of Bcl-3 leads to an increase in Hdm2 levels and to a subsequent loss of DNA damage-induced p53 protein stability. The consequence of the Bcl-3-mediated inhibition of p53 is a loss of induction of p53 target genes and protection against DNA damage-induced apoptosis. Interestingly, the loss of Bcl-3 leads to the inability of p53 to induce hdm2 transcription and, correspondingly, to enhanced p53 protein stability, suggesting that a normal physiological role of Bcl-3 is to limit the p53 activation response.

Bcl-3 overexpression is characteristic of a growing number of human cancers (Cogswell et al. 2000; Thornburg et al. 2003; Canoz et al. 2004), yet a specific oncogenic role for Bcl-3 in these cancers has been elusive. Recently we and others (Westerheide et al. 2001; Rocha et al. 2003) demonstrated that Bcl-3 can contribute to cyclin D1 transcription, a key component in driving cell cycle progression. The finding that Bcl-3 can inhibit p53 function provides a novel mechanism for how aberrant Bcl-3 expression can fulfill an oncogenic function. The importance of p53 as a tumor suppressor is illustrated by the fact that it is mutated or lost in over half of all human cancers tested (Vousden and Lu 2002; Vousden and Prives 2005), and presumably cancer cells with wild-type p53 have compromised its function through different mechanisms. Loss of the tumor suppressor ARF and high level expression of Hdm2 have been thought to be two of main ways that this is achieved. Consistent with this, it has been reported (Bond et al. 2004) that an Hdm2 promoter polymorphism increases affinity for the transcription factor Sp1 promoting high level transcription and subsequent attenuation of the p53 response. In addition, N-Myc (Slack et al. 2005) and estrogen receptor-α (Phelps et al. 2003) can regulate Hdm2 in a p53-independent fashion. Similarly, Bcl-3, through induction of Hdm2, would provide a level of inhibition to p53 that tumor cells need to progress, bypassing the need for mutation or deletion of p53.

Current models of p53 activation by DNA damage suggest that post-translational modification of p53 disrupts its interaction with Hdm2, and thus it might be suggested that any increase in Hdm2 protein levels would have a minimal effect on p53 protein stability following

DNA damage. However, it is likely that the dynamics of the DNA damage response are more complex. First, it has been reported that modification of both p53 and Hdm2 is important for the disruption of the interaction (L.D. Mayo et al. 1997; Hay and Meek 2000), suggesting that a consistent supply of newly transcribed Hdm2 induced by Bcl-3 would provide a steady stream of unmodified Hdm2 to re-engage p53. Further, the fact that p53-induced Hdm2 provides negative feedback regulation of p53 suggests that at some point following the initial damage event, the newly transcribed Hdm2 is able to rebind to p53 and turn off the response. Consistent with this, our data indicate that Bcl-3-induced Hdm2 will indeed have the effect of dampening the p53 response. This conclusion is further supported by the many reports of Hdm2 overexpression in human cancers (Momand et al. 1998).

Furthermore, while our data indicate that Bcl-3 can regulate p53 through induction of Hdm2, we cannot rule out additional roles for Bcl-3 in regulating p53 function. For example, certain p53-dependent genes have NF-κB consensus sites in their promoters, and Bcl-3, presumably through interactions with the p50 or p52 NF-κB subunits, may play a direct role in the regulation of those genes. In fact, we have found recruitment of Bcl-3 as well as other NF-κB subunits to a number of those promoters and are currently investigating those mechanisms (D. Kashatus, unpubl.). In addition, higher levels of Bcl-3 may affect NF-κB dimer composition and indirectly affect NF-κB regulation of those promoters. It is also possible that Bcl-3 regulates p53 protein levels or functional activity through additional mechanisms. While we find no evidence that Bcl-3 has a role in direct transcriptional regulation of p53, it may affect p53 through control of stability or translational efficacy.

The finding of this novel function for Bcl-3 raises intriguing questions regarding a role for Bcl-3 in development, immune and inflammatory function, and the stress response. An indication of what that role may be comes from a the recent finding that Bcl-6, a transcriptional repressor also found aberrantly expressed in B-cell lymphomas that is unrelated to Bcl-3, directly inhibits p53 transcription in germinal center B cells (Phan and Dalla-Favera 2004). This negative regulation of p53 is presumed to be important to allow these cells to tolerate the DNA damage required for immunoglobulin class switch recombination and somatic hypermutation without mounting an apoptotic response. Our new data, plus the fact that Bcl-3 knockout mice, like the Bcl-6 knockouts, fail to properly develop germinal centers (Franzoso et al. 1997), suggest that Bcl-3 may function to block p53 activation in germinal center B cells, but through a different mechanism than that reported for Bcl-6. It has also been shown that expression of Bcl-3 correlates with survival in adjuvant-induced T cells (Mitchell et al. 2001). Activated T cells require a survival signal following antigen stimulation in order to avoid apoptosis and to undergo clonal expansion. The apoptotic signal has been shown to be mediated by the p53 family member p73 (Wan and DeGregori 2003). Given its role in p53 inhibition, Bcl-3 may protect activated T cells through down-regulation of this apoptotic response by inhibiting p73 through a similar mechanism. The failure of Bcl-3-null mice to mount an effective T-cell response to influenza virus supports this role for Bcl-3 (Franzoso et al. 1997). Finally, we observe that Bcl-3 is activated by UV, potentially at the level of protein stabilization (Fig. 2). Given that loss of Bcl-3 leads to enhanced p53 activation and to enhanced apoptosis (Fig. 3), it is likely that the induction of Bcl-3 by UV (or by other DNA damaging agents) functions normally to limit p53 induction and apoptosis.

Given the previous intriguing results regarding both cooperative (Ryan et al. 2000) and antagonistic (Webster and Perkins 1999) relationships between the NF-κB and p53 pathways, it would be expected that the findings reported here relate to these original findings. However, the direct relationship between Bcl-3 expression and the classic NF-κB pathway remains unclear. In this regard, it has been reported that Bcl-3 gene expression can be induced in an NF-κB-dependent manner (Brasier et al. 2001). Our analyses of p65 null MEFs, however, do not reveal a similar defect in mdm2 expression or p53 stabilization (D. Kashatus, unpubl.), suggesting the Bcl-3-mediated regulation is p65 independent. Further analysis of cells lacking other NF-κB subunits, specifically p50 and p52, should allow us to determine their requirement in this regulation. The fact that loss of IKKβ leads to a loss of mdm2 induction suggests that this kinase may function upstream of Bcl-3 in this response (Tergaonkar et al. 2002). IKKβ may act through regulation of p50 or p52, or it could potentially be involved in the DNA damage-induced stabilization of Bcl-3 through direct phosphorylation.

In summary, our findings provide a critical understanding of the functional consequences of Bcl-3 expression in human cancer cells and could potentially have direct clinical applications for cancer treatment. Unlike Bcl-3, p53 and Hdm2 are popular targets for drug development, and these findings may provide rationale for a potential new intervention pathway for certain cancers. These data also provide an additional link in the expanding relationships between the NF-κB/IκB family and p53, and open new avenues for exploration into how these two important protein families interact to control oncogenesis.

Materials and methods

Cell culture

Primary murine embryo fibroblasts were isolated from day 13 embryos and grown in DMEM supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1× penicillin/streptomycin (GIBCO). MCF-7 cells (ATCC) were grown in MEMα supplemented with 10% FBS, 10 μg/mL insulin (GIBCO), 1 mM sodium pyruvate (GIBCO), and 1× penicillin/streptomycin. Karpas 299 cells (DSMZ) were grown in RPMI supplemented with 10% FBS and 1× penicillin/streptomycin. HT1080 cells (ATCC) were grown in DMEM supplemented with 10% FBS and 1× penicillin/streptomycin. U-2OS cells (ATCC) were grown in McCoys

5A supplemented with 15% FBS and 1× penicillin/streptomycin. MCF-7B cells were generated by transfecting the expression construct pFlag-Bcl-3 into MCF-7 cells. Stable clones were generated in medium containing 1 µg/mL puromycin (Sigma). Clones were verified by Western blotting with a Bcl-3-specific antibody. Cisplatin (Sigma) was resuspended in DMSO and stored at –20°C. RITA was resuspended in DMSO to a concentration of 10 mM and stored in the dark at –20°C. Pifithrin-α (A.G. Scientific) was resuspended in DMSO to a concentration of 10 mM and stored in the dark at –20°C. Staurosporine (Sigma) was resuspended in DMSO to a concentration of 2 mM and stored at –20°C. For UV treatments, cells were placed under a UV lamp and dosage was measured with a UV-X radiometer (Ultra Violet Products).

Antibodies

For Western blots, ChIP analysis, and supershift analysis, we used antibodies against p53 (FL393, Santa Cruz), Bcl-3 (Upstate), Hdm2 (Ab-1, Calbiochem), mdm2 (2A10, from A. Levine, Institute for Advanced Study, Princeton, NJ), Flag (M2, Sigma), p65 (5192, Rockland), p52 (from N. Rice, National Cancer Institute, Frederick, MD), p50 (H-119, Santa Cruz), and β-tubulin (H-235, Santa Cruz). HRP-conjugated anti-mouse and anti-rabbit IgG secondary antibodies were from Promega.

Western blot

Typically, cells were plated in a 100-mm or 6-well tissue culture dish and treated as indicated. After the indicated time points, cells were wash with PBS and lysed in modified RIPA buffer (1% NP40, 20 mM Tris, 137 mM NaCl, 10% glycerol, 2 mM EDTA, 2 µg/mL aprotinin, 2 µg/mL leupeptin, 1 mM PMSF); 10 or 15 µg of protein was loaded onto 4%–12% Bis-Tris gels (Invitrogen). After electrophoresis, gels were transferred to nitrocellulose (Bio-Rad) and blocked for 1 h in TBS containing 0.5% Tween-20, 4% milk, and 1% BSA. Primary and secondary antibody incubations were performed in blocking buffer at 4°C (primary) or room temperature (secondary).

Apoptosis detection

Cells were plated in 100-mm dishes and treated with the indicated dose of UV or cisplatin. At the indicated time points, cells were washed with PBS and collected by trypsinization. Cells were washed again in PBS and resuspended in 100 µL annexin-binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$ at pH 7.4) containing 5 µL Alexa-488 conjugated Annexin-V (Molecular Probes) plus 1 µg/mL propidium iodide (Sigma). Cells were incubated for 15 min at room temperature and then analyzed on a Facscalibur (Becton Dickinson). Apoptotic cells were measured as positive for Annexin-V staining but negative for propidium iodide.

Plasmid constructs

pCMV2–Flag-Bcl-3 (Westerheide et al. 2001), pCMV–Flag-p53 (Zhang et al. 1998), pg-13–luciferase (el-Deiry et al. 1993), and Hdm2–Luc01, Hdm2–Luc02, and Hdm2–Luc03 (Phelps et al. 2003) were described previously.

ChIP assay

ChIP analysis was performed using a ChIP kit (Upstate Biotechnology) and a modified version of the manufacturer's protocol. Following the indicated treatment, cells were fixed for 5 min in 1% formaldehyde, washed with PBS, and lysed for 10 min in lysis buffer. Chromatin was sheared by sonication to an average size of ~1 kb and precleared for 2 h at 4°C with salmon sperm DNA-saturated protein G-Sepharose beads. Chromatin solutions were precipitated overnight at 4°C using 10 µL of the indicated antibodies. Immune complexes were collected with salmon sperm DNA-saturated protein G-Sepharose beads for 1 h and washed extensively following the manufacturer's protocol. Input and immunoprecipitated chromatin were incubated overnight at 65°C to reverse cross-links. After proteinase K digestion, DNA was extracted with phenol/chloroform and precipitated with ethanol. Precipitated DNAs were analyzed by real-time PCR on an ABI 7100 using SYBR green master mix (ABI). Each sample was normalized to input using the $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen 2001). Hdm2 P2 promoter-specific primers used were 5′-GAGGTCCGGATGATCGCAGG-3′ and 5′-GTGGCGTGCGTCCGTGCCCA-3′, and β-actin promoter-specific primers used were 5′-CCTCCTCCTTCTTCCTCA ATCT-3′ and 5′-GGGGAGAGGGGAGGAAA-3′.

Luciferase assays

Typically, cells were plated in 24-well dishes and allowed to grow to ~70% confluency. Cells were transfected with the indicated plasmids using polyfect reagent (Qiagen) following the manufacturer's protocol. Forty-eight hours post-transfection, extracts were prepared using the Dual Luciferase Assay System (Promega) following the manufacturer's protocol, and luciferase activity was measured on an LMax luminometer (Molecular Devices).

Real-time PCR

Cells were plated in 100-mm dishes and treated with the indicated doses of UV or cisplatin. At the indicated time points, cells were washed in PBS and lysed in Trizol Reagent (Invitrogen), and RNA was collected following the manufacturer's protocol. cDNA was generated using the M-MLV reverse transcriptase kit (Invitrogen), and quantitative PCR was performed on an ABI Prism 7000 (Applied Biosystems) using gene-specific TAQman primer/probe sets (Applied Biosystems).

RNAi

Synthetic double-stranded RNA (dsRNA) oligonucleotides targeting Bcl-3 and Hdm2 were purchased from Xeragon and Ambion, respectively. The targeted sequence for Bcl-3 is 5′-AATG GTCTTCTCTCCGCATCA-3′. Cells were plated in six-well dishes and allowed to grow to ~70% confluency. Transfection of the gene specific siRNA plus a control siRNA (Ambion) was performed using the transmessenger transfection reagent (Ambion) according to the manufacturer's protocol. Forty-eight hours post-transfection, cells were treated for the indicated time points and lysed, and Western analysis was performed as described.

Electrophoretic mobility shift assay (EMSA)

Nuclear and cytoplasmic extracts were obtained from MCF-7 and MCF-7B cells, and gel shift analysis was performed as previously described (M.W. Mayo et al. 1997). Briefly, an oligonucleotide corresponding to the putative NF-κB site in the *Hdm2* P2 promoter (5′-GGTGGTTCGGAGGTCTCCGCGG GAGT-3′) was radiolabeled using [α-^{32}P]dCTP (Perkin Elmer). The probe was incubated with 4 µg of nuclear extract and 0.1µg/µL poly dIdC in DNA-binding buffer (50 mM NaCl, 10 mM Tris at pH 7.6, 10% glycerol, 1 mM DTT, 0.5 mM EDTA) for 15 min

at room temperature. For antibody supershift analysis, extracts were preincubated for 15 min at room temperature with 1 µg of antiserum before the addition of the radiolabeled gel shift probe. Reactions were separated using nondenaturing PAGE and visualized by autoradiography.

Acknowledgments

We thank Dr. Arnold Levine for providing the mdm2 antibody, Dr. Galina Selivanova for providing the RITA compound, Dr. Jeremy Blaydes for providing the Hdm2 promoter luciferase constructs, Dr. Nancy Rice for providing the p52 antibody, Dr. Yue Xiong for providing the pCMV–p53 construct, Dr. Ned Sharpless for providing the pg-13 luciferase construct and for helpful discussions, and Dr. Kris Steinbrecher for help generating MEFs. Research support was provided by NIH grants AI35098, CA73756, and CA75080 to A.S.B; by the Department of Defense (DAMD 17-03-1-0401) to A.S.B.; and by the UNC Breast Cancer SPORE program. Additional support was provided by the Waxman Cancer Research Foundation. The authors have no conflict of interest relative to the data reported in this manuscript.

References

Barak, Y., Juven, T., Haffner, R., and Oren, M. 1993. mdm2 expression is induced by wild type p53 activity. *EMBO J.* **12:** 461–468.

Bond, G.L., Hu, W., Bond, E.E., Robins, H., Lutzker, S.G., Arva, N.C., Bargonetti, J., Bartel, F., Taubert, H., Wuerl, P., et al. 2004. A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. *Cell* **119:** 591–602.

Bond, G.L., Hu, W., and Levine, A.J. 2005. MDM2 is a central node in the p53 pathway: 12 years and counting. *Curr. Cancer Drug Targets* **5:** 3–8.

Boulton, S.J., Gartner, A., Reboul, J., Vaglio, P., Dyson, N., Hill, D.E., and Vidal, M. 2002. Combined functional genomic maps of the *C. elegans* DNA damage response. *Science* **295:** 127–131.

Bours, V., Franzoso, G., Azarenko, V., Park, S., Kanno, T., Brown, K., and Siebenlist, U. 1993. The oncoprotein Bcl-3 directly transactivates through κ B motifs via association with DNA-binding p50B homodimers. *Cell* **72:** 729–739.

Brasier, A.R., Lu, M., Hai, T., Lu, Y., and Boldogh, I. 2001. NF-κ B-inducible BCL-3 expression is an autoregulatory loop controlling nuclear p50/NF-κ B1 residence. *J. Biol. Chem.* **276:** 32080–32093.

Canoz, O., Rassidakis, G.Z., Admirand, J.H., and Medeiros, L.J. 2004. Immunohistochemical detection of BCL-3 in lymphoid neoplasms: A survey of 353 cases. *Mod. Pathol.* **17:** 911–917.

Chipuk, J.E., Kuwana, T., Bouchier-Hayes, L., Droin, N.M., Newmeyer, D.D., Schuler, M., and Green, D.R. 2004. Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. *Science* **303:** 1010–1014.

Cogswell, P.C., Guttridge, D.C., Funkhouser, W.K., and Baldwin Jr., A.S. 2000. Selective activation of NF-κ B subunits in human breast cancer: Potential roles for NF-κ B2/p52 and for Bcl-3. *Oncogene* **19:** 1123–1131.

Dechend, R., Hirano, F., Lehmann, K., Heissmeyer, V., Ansieau, S., Wulczyn, F.G., Scheidereit, C., and Leutz, A. 1999. The Bcl-3 oncoprotein acts as a bridging factor between NF-κB/Rel and nuclear co-regulators. *Oncogene* **18:** 3316–3323.

el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. 1993. WAF1, a potential mediator of p53 tumor suppression. *Cell* **75:** 817–825.

Espinosa, J.M., Verdun, R.E., and Emerson, B.M. 2003. p53 functions through stress- and promoter-specific recruitment of transcription initiation components before and after DNA damage. *Mol. Cell* **12:** 1015–1027.

Franzoso, G., Carlson, L., Scharton-Kersten, T., Shores, E.W., Epstein, S., Grinberg, A., Tran, T., Shacter, E., Leonardi, A., Anver, M., et al. 1997. Critical roles for the Bcl-3 oncoprotein in T cell-mediated immunity, splenic microarchitecture, and germinal center reactions. *Immunity* **6:** 479–490.

Ghosh, S. and Karin, M. 2002. Missing pieces in the NF-κB puzzle. *Cell* **109(Suppl):** S81–S96.

Hay, T.J. and Meek, D.W. 2000. Multiple sites of in vivo phosphorylation in the MDM2 oncoprotein cluster within two important functional domains. *FEBS Lett.* **478:** 183–186.

Hayden, M.S. and Ghosh, S. 2004. Signaling to NF-κB. *Genes & Dev.* **18:** 2195–2224.

Hubinger, G., Muller, E., Scheffrahn, I., Schneider, C., Hildt, E., Singer, B.B., Sigg, I., Graf, J., and Bergmann, L. 2001. CD30-mediated cell cycle arrest associated with induced expression of p21(CIP1/WAF1) in the anaplastic large cell lymphoma cell line Karpas 299. *Oncogene* **20:** 590–598.

Issaeva, N., Bozko, P., Enge, M., Protopopova, M., Verhoef, L.G., Masucci, M., Pramanik, A., and Selivanova, G. 2004. Small molecule RITA binds to p53, blocks p53–HDM-2 interaction and activates p53 function in tumors. *Nat. Med.* **10:** 1321–1328.

Jeffers, J.R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K.H., Han, J., Chittenden, T., Ihle, J.N., et al. 2003. Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. *Cancer Cell* **4:** 321–328.

Komarov, P.G., Komarova, E.A., Kondratov, R.V., Christov-Tselkov, K., Coon, J.S., Chernov, M.V., and Gudkov, A.V. 1999. A chemical inhibitor of p53 that protects mice from the side effects of cancer therapy. *Science* **285:** 1733–1737.

Kucharczak, J., Simmons, M.J., Fan, Y., and Gelinas, C. 2003. To be, or not to be: NF-κB is the answer: Role of Rel/NF-κB in the regulation of apoptosis. *Oncogene* **22:** 8961–8982.

Livak, K.J. and Schmittgen, T.D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta(T)}$ method. *Methods* **25:** 402–408.

Mayo, L.D., Turchi, J.J., and Berberich, S.J. 1997. Mdm-2 phosphorylation by DNA-dependent protein kinase prevents interaction with p53. *Cancer Res.* **57:** 5013–5016.

Mayo, M.W., Wang, C.Y., Cogswell, P.C., Rogers-Graham, K.S., Lowe, S.W., Der, C.J., and Baldwin Jr., A.S. 1997. Requirement of NF-κB activation to suppress p53-independent apoptosis induced by oncogenic Ras. *Science* **278:** 1812–1815.

McKeithan, T.W., Rowley, J.D., Shows, T.B., and Diaz, M.O. 1987. Cloning of the chromosome translocation breakpoint junction of the t(14;19) in chronic lymphocytic leukemia. *Proc. Natl. Acad. Sci.* **84:** 9257–9260.

Mitchell, T.C., Hildeman, D., Kedl, R.M., Teague, T.K., Schaefer, B.C., White, J., Zhu, Y., Kappler, J., and Marrack, P. 2001. Immunological adjuvants promote activated T cell survival via induction of Bcl-3. *Nat. Immunol.* **2:** 397–402.

Momand, J., Jung, D., Wilczynski, S., and Niland, J. 1998. The MDM2 gene amplification database. *Nucleic Acids Res.* **26:** 3453–3459.

Na, S.Y., Choi, H.S., Kim, J.W., Na, D.S., and Lee, J.W. 1998. Bcl3, an IκB protein, as a novel transcription coactivator of the retinoid X receptor. *J. Biol. Chem.* **273:** 30933–30938.

Nakano, K. and Vousden, K.H. 2001. PUMA, a novel proapo-

ptotic gene, is induced by p53. *Mol. Cell* **7:** 683–694.
Nishikori, M., Maesako, Y., Ueda, C., Kurata, M., Uchiyama, T., and Ohno, H. 2003. High-level expression of BCL3 differentiates t(2;5)(p23;q35)-positive anaplastic large cell lymphoma from Hodgkin disease. *Blood* **101:** 2789–2796.
Nolan, G.P., Fujita, T., Bhatia, K., Huppi, C., Liou, H.C., Scott, M.L., and Baltimore, D. 1993. The bcl-3 proto-oncogene encodes a nuclear I κ B-like molecule that preferentially interacts with NF-κ B p50 and p52 in a phosphorylation-dependent manner. *Mol. Cell. Biol.* **13:** 3557–3566.
Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. 2000. Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. *Science* **288:** 1053–1058.
Phan, R.T. and Dalla-Favera, R. 2004. The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells. *Nature* **432:** 635–639.
Phelps, M., Darley, M., Primrose, J.N., and Blaydes, J.P. 2003. p53-independent activation of the hdm2-P2 promoter through multiple transcription factor response elements results in elevated hdm2 expression in estrogen receptor α-positive breast cancer cells. *Cancer Res.* **63:** 2616–2623.
Rocha, S., Martin, A.M., Meek, D.W., and Perkins, N.D. 2003. p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing increased association of the p52 NF-κB subunit with histone deacetylase 1. *Mol. Cell. Biol.* **23:** 4713–4727.
Ryan, K.M., Ernst, M.K., Rice, N.R., and Vousden, K.H. 2000. Role of NF-κB in p53-mediated programmed cell death. *Nature* **404:** 892–897.
Schwarz, E.M., Krimpenfort, P., Berns, A., and Verma, I.M. 1997. Immunological defects in mice with a targeted disruption in Bcl-3. *Genes & Dev.* **11:** 187–197.
Slack, A., Chen, Z., Tonelli, R., Pule, M., Hunt, L., Pession, A., and Shohet, J.M. 2005. The p53 regulatory gene MDM2 is a direct transcriptional target of MYCN in neuroblastoma. *Proc. Natl. Acad. Sci.* **102:** 731–736.
Suzuki, K., Yokoyama, S., Waseda, S., Kodama, S., and Watanabe, M. 2003. Delayed reactivation of p53 in the progeny of cells surviving ionizing radiation. *Cancer Res.* **63:** 936–941.
Tergaonkar, V., Pando, M., Vafa, O., Wahl, G., and Verma, I. 2002. p53 stabilization is decreased upon NFκB activation: A role for NFκB in acquisition of resistance to chemotherapy. *Cancer Cell* **1:** 493–503.
Thornburg, N.J., Pathmanathan, R., and Raab-Traub, N. 2003. Activation of nuclear factor-κB p50 homodimer/Bcl-3 complexes in nasopharyngeal carcinoma. *Cancer Res.* **63:** 8293–8301.
Viatour, P., Bentires-Alj, M., Chariot, A., Deregowski, V., de Leval, L., Merville, M.P., and Bours, V. 2003. NF-κ B2/p100 induces Bcl-2 expression. *Leukemia* **17:** 1349–1356.
Viatour, P., Dejardin, E., Warnier, M., Lair, F., Claudio, E., Bureau, F., Marine, J.C., Merville, M.P., Maurer, U., Green, D., et al. 2004. GSK3-mediated BCL-3 phosphorylation modulates its degradation and its oncogenicity. *Mol. Cell* **16:** 35–45.
Villunger, A., Michalak, E.M., Coultas, L., Mullauer, F., Bock, G., Ausserlechner, M.J., Adams, J.M., and Strasser, A. 2003. p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. *Science* **302:** 1036–1038.
Vousden, K.H. and Lu, X. 2002. Live or let die: The cell's response to p53. *Nat. Rev. Cancer* **2:** 594–604.
Vousden, K.H. and Prives, C. 2005. P53 and prognosis: New insights and further complexity. *Cell* **120:** 7–10.
Wan, Y.Y. and DeGregori, J. 2003. The survival of antigen-stimulated T cells requires NFκB-mediated inhibition of p73 expression. *Immunity* **18:** 331–342.
Watanabe, N., Wachi, S., and Fujita, T. 2003. Identification and characterization of BCL-3-binding protein: Implications for transcription and DNA repair or recombination. *J. Biol. Chem.* **278:** 26102–26110.
Webster, G.A. and Perkins, N.D. 1999. Transcriptional cross talk between NF-κB and p53. *Mol. Cell Biol.* **19:** 3485–3495.
Wessells, J., Baer, M., Young, H.A., Claudio, E., Brown, K., Siebenlist, U., and Johnson, P.F. 2004. BCL-3 and NF-κB p50 attenuate lipopolysaccharide-induced inflammatory responses in macrophages. *J. Biol. Chem.* **279:** 49995–50003.
Westerheide, S.D., Mayo, M.W., Anest, V., Hanson, J.L., and Baldwin Jr., A.S. 2001. The putative oncoprotein Bcl-3 induces cyclin D1 to stimulate G(1) transition. *Mol. Cell. Biol.* **21:** 8428–8436.
Yu, J., Zhang, L., Hwang, P.M., Kinzler, K.W., and Vogelstein, B. 2001. PUMA induces the rapid apoptosis of colorectal cancer cells. *Mol. Cell* **7:** 673–682.
Zhang, Y., Xiong, Y., and Yarbrough, W.G. 1998. ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. *Cell* **92:** 725–734.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 281, NO. 37, pp. 26976–26984, September 15, 2006

IKK-i/IKKε Controls Constitutive, Cancer Cell-associated NF-κB Activity via Regulation of Ser-536 p65/RelA Phosphorylation*

Received for publication, April 3, 2006, and in revised form, June 19, 2006 Published, JBC Papers in Press, July 13, 2006, DOI 10.1074/jbc.M603133200

Mazhar Adli and Albert S. Baldwin[1]

From the Lineberger Comprehensive Cancer Center, Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599

Nuclear factor κB (NF-κB) has been studied extensively as an inducible transcriptional regulator of the immune and inflammatory response. NF-κB activation downstream of lipopolysaccharide or cytokine stimulation is controlled by the IκB kinase complex, which contains IKKα and IKKβ. Significantly, the constitutive activity of NF-κB has been implicated as an important aspect of many cancer cells, but mechanisms associated with this activity are poorly understood. An inducible kinase, IKK-i/IKKε, related to the catalytic forms of the IκB kinase, has been studied as an anti-viral, innate immune regulator through its ability to control the activity of the transcription factors IRF-3 and IRF-7. Here, we demonstrate that IKK-i/IKKε is expressed in a number of cancer cells and is involved in regulating NF-κB activity through its ability to control basal/constitutive, but not cytokine-induced, p65/RelA phosphorylation at Ser-536, a modification proposed to contribute to the transactivation function of NF-κB. Knockdown of IKK-i/IKKε or expression of a S536A mutant form of p65 suppresses HeLa cell proliferation. The data indicate a role for IKK-i/IKKε in controlling proliferation of certain cancer cells through regulation of constitutive NF-κB activity.

The transcription factor nuclear factor-κB (NF-κB)[2] plays a pivotal role in controlling the expression of a diverse set of genes that contribute to a variety of biological functions, including cell survival, cell proliferation, and immune and inflammatory responses (1). The classic form of NF-κB is composed of a heterodimer of the p50 and p65 subunits, which is preferentially localized in the cytoplasm as an inactive complex with inhibitor proteins of the IκB family. Following exposure of cells to a variety of stimuli, including inflammatory cytokines and LPS, IκBs are phosphorylated by the IKKα/β complex, polyubiquitinated, and subsequently degraded by the 26 S proteasome complex (1–3). Released NF-κB complexes then accumulate in the nucleus, where they transcriptionally regulate the expression of genes involved in the immune and inflammatory responses (3).

Based on a number of observations, it was assumed that virtually all inducers of NF-κB lead to the activation of a single classic IKKα/β/γ complex. However, recent studies demonstrated the existence of distinct IKK complexes that do not contain IKKα, -β, or -γ (4). One of these complexes was described as a PMA-inducible IκB kinase complex, with a critical component being an IKK-related kinase designated IKKε (5), which is identical to a kinase named IKK-i identified via its induction downstream of LPS-induced signaling (6). IKKε in turn is closely related to another recently discovered IKK-related kinase designated as TBK1 (TANK-binding kinase 1) (7) or NAK (NF-κB activating kinase) (8). TBK1, which is highly homologous to IKKε, binds to TANK and TRAF and may form an alternative IKK complex consisting of IKKε and TBK1 (7).

IKKε and TBK1 are enzymatically distinct from the homologous enzymes IKKα and IKKβ (9) and have been shown to play important roles in the innate immune response. These kinases function as critical components of the interferon regulatory factor 3 (IRF3) and IRF7 signaling pathways involved in responses to viral infection or dsRNA treatment (10, 11). Recent studies demonstrated that embryonic fibroblasts (MEFs) derived from TBK1-deficient ($TBK1^{-/-}$) mice show impaired production of NF-κB-dependent (12) as well as IRF3-dependent (13) gene expression. It has also been shown that IFN-β and IFN-inducible gene expression is defective in TBK1 knock-out cells in response to LPS, poly(I:C), or viral infection (14–16).

The relationships of IKKε and TBK1 with NF-κB activation remain enigmatic. Although recent studies defined their roles in IRF3 and IRF7 transcriptional activation (10, 11, 13) and suggested their involvement in NF-κB activation (7–9, 12, 17, 18), the exact molecular mechanism of NF-κB activation by these kinases is not clearly understood. One report (19) indicated that IKKε plays a key role integrating signals induced by pro-inflammatory stimuli by activating CAAT/enhancer-binding protein δ whose expression is regulated by NF-κB. There is a recent report suggesting that IKKε and TBK1 are among the

* This work was supported by National Institutes of Health Grants AI35098, CA73756, and CA75080 (to A. S. B.) and by the Waxman Cancer Research Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed: 22-000 Lineberger Cancer Center, CB #7295, University of North Carolina School of Medicine, Chapel Hill, NC 27599. Tel.: 919-966-3652; Fax: 919-966-0444; E-mail: abaldwin@med.unc.edu.

[2] The abbreviations used are: NF-κB, nuclear factor κB; LPS, lipopolysaccharide; PMA, phorbol 12-myristate 13-acetate; IRF, interferon regulatory factor; MEF, embryonic fibroblast; IFN, interferon; TNF, tumor necrosis factor; GFP, green fluorescent protein; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; EMSA, electrophoretic mobility shift assay; siRNA, small interference RNA; shRNA, small hairpin RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DKO, IKKε/TBK1 doubly deficient MEFs; WT, wild type; KM, kinase mutant.

kinases that mediate inducible phosphorylation of p65 at Ser-536 (19), an event proposed to stimulate inherent p65 transactivation function (20). In this model, TBK1 and IKKε would control NF-κB at a level distinct from the traditional IKK-mediated control of IκB degradation.

Here, we show that IKKε is expressed in a variety of cancer cell lines. Based on this, we have investigated a role for IKKε as related to constitutive, cancer-associated NF-κB activity. Our experiments reveal an important role for IKKε in controlling the activation of Ser-536 phosphorylation of the RelA/p65 subunit and functional NF-κB activity in several cancer cell lines and in 293T cells.

MATERIALS AND METHODS

Reagents and Materials—All cells were cultured in Dulbecco's modified Eagle's medium, complemented with 10% fetal calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin. Generation of wild-type, IKKε single, and IKKε-TBK1 double knock-out cells was described previously (15), and they were the kind gift of S. Akira. A monoclonal antibody against FLAG (M2) was obtained from Sigma. An antibody to IKKε and to phospho-specific NF-κB p65 (Ser-536) were obtained from Cell Signaling. Antibodies to β-tubulin and to IκBα were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to IKKα and IKKβ were obtained from Upstate Biotechnology Inc. LPS (L6529, Sigma) was used at a final concentration of 1 μg/ml. Recombinant human TNF-α (Promega) was used at a final concentration of 10 ng/ml. Recombinant mouse interleukin-1β (Roche Applied Sciences) was used at a final concentration of 10 ng/ml. Effectene transfection reagent obtained from Qiagen was used according to the manufacturer's protocol.

Plasmids and Constructs—3X-κB luciferase reporter construct contains 3 κB consensus DNA binding sites from the major histocompatibility complex class I promoter fused upstream of firefly luciferase. Wild-type and kinase mutant forms of FLAG-tagged IKKε (IKKε K38) have been described previously (5). Wild-type and kinase mutant forms of TBK1 (TBK1 K38) have been described previously (11) and were the kind gift of J. Hiscott. pLuc-110 IFNβ reporter constructs have been described previously (10) and were the kind gifts of T. Maniatis.

Transfections and Reporter Assays—For transient transfections, the indicated cell lines were seeded in 6-well plates at 30–50% density and transfected the next day with the indicated expression vectors for 48 h using Effectene (Qiagen) transfection reagent according to the manufacturer's instruction. For reporter assays, 2×10^5 cells were seeded in 24-well plates and co-transfected the next day with the indicated luciferase reporter genes and a β-galactosidase reporter gene as an internal control. The total amount of transfected DNA (500 ng of DNA) in each well was adjusted by adding empty plasmid vector (pcDNA3.1). Luciferase activity of whole cell lysates was measured by using a luciferase assay kit (Promega). β-Galactosidase activity was measured by liquid β-galactosidase assay with chlorophenolred-β-D-galactopyranoside substrate. Relative luciferase activity was calculated by normalizing the assay results to β-galactosidase expression values. Luciferase -fold induction was calculated by normalizing the results to control treatment, which was assumed as 1 -fold induction.

Western Blot—After stimulation, cultured cells were lysed on ice for 5 min in radioimmune precipitation assay lysis buffer with freshly added protease and phosphatase inhibitor cocktails. Lysates were cleared by centrifugation at 4 °C for 15 min at 13,000 × *g*. The amount of total protein was measured, and equal amounts (20 μg) were fractionated by NuPAGE Novex 4–12% Bis-Tris gels (Invitrogen) and electrotransferred to polyvinylidene difluoride membranes. Membranes were blotted with the indicated antibodies, and proteins were detected using an enhanced chemiluminescence detection system (Amersham Biosciences). Where indicated, membranes were stripped and re-probed with the indicated antibodies.

Electrophoretic Mobility Shift Assay—EMSAs were performed as previously described (21). Briefly, 4–5 μg of nuclear extracts prepared following cell stimulation was incubated with a radiolabeled DNA probe containing an NF-κB consensus site. For supershifts, 1 μl of anti-p65 antibody (Rockland) or 2 μl of anti-p50 antibody (Santa Cruz Biotechnology, SC-7178) was added, and the binding reaction was allowed to proceed for an additional 15 min. Protein-DNA complexes were resolved on a non-denaturing polyacrylamide gel and visualized by autoradiography.

siRNA and shRNA Transfection—IKKε mRNA was knocked down with the GeneSupressor System (IMGENEX). Plasmids encoding control shRNA and IKKε shRNA were transfected by Effectene transfection reagent (Qiagen) according to manufacturer's instructions for 48–72 h. Additionally, Silencer® predesigned siRNA targeting IKKε and TBK1 have also been utilized and were transfected with a Silencer® siRNA Transfection II kit. Lysate preparation and Western blots were performed as described.

Cell Proliferation and MTT Assay—A cell proliferation assay has been performed as described by using the TACS MTT assay kit (R&D Systems). First, the optimal cell number, which was 1×10^4 for HeLa cells in our system, was determined. After transfection, an equal number of cells was seeded in a 96-well plate for the indicated times. 10 μl of MTT reagent was added to each well, including the blank wells, and the mixture was incubated for 4–5 h at 37 °C. Then 100 μl of detergent reagent was added, the mixture was incubated for at least 12 h at 37 °C, and absorbance values at 570 nm were measured with a reference wavelength of 650 nm.

RESULTS

IKKε Is Expressed in a Number of Cancer Cells and in SV40 Large T-immortalized 293 Cells—To address a potential role for IKKε in controlling NF-κB activity, we explored the expression of IKKε in a variety of cell lines. Immunoblotting of extracts of several cancer cell lines revealed constitutive expression of IKKε in breast cancer cell lines MDA MB 468, SK BR3, Sum 226, and MCF7; HeLa cells; PC3 and LNCaP prostate cancer cells; and 293T embryonic kidney cells (Fig. 1). As a marker for endogenous NF-κB activity, we immunoblotted the extracts from these cells with an antibody that recognizes only the RelA/p65 subunit phosphorylated at Ser-536. These data revealed a correlation between IKKε expression and phosphorylated Ser-

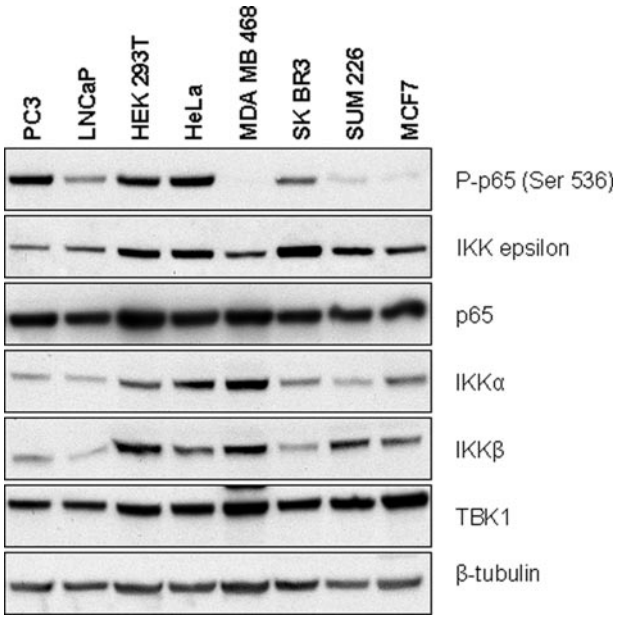


FIGURE 1. **IKKε expression is elevated in a number of cancer cells.** Total cell extracts from indicated cell lines were prepared and immunoblotted with the indicated antibodies. IKKε expression was elevated in many cancers cells and the level of the expression is well correlated with p65 phosphorylation at Ser-536. The levels of IKKα, IKKβ, and TBK1 are not consistently correlated with p65 phosphorylation level.

536 RelA/p65 in most of the cells analyzed. Notably, TBK1 was expressed in these cells but did not consistently correlate with RelA/p65 Ser-536 phosphorylation. The data also reveal that IKKε, although considered an inducible kinase, is found to be constitutively expressed at significant levels in most of the cell lines investigated.

IKKε or TBK1 Activates an NF-κB-dependent Reporter in a Kinase-dependent Manner—To investigate a potential role for IKKε and TBK1 in NF-κB regulation, experiments were initiated to analyze their potential involvement in controlling NF-κB-dependent promoters. Based on the results from Fig. 1, we focused these experiments on HEK 293T cells, because IKKε is expressed and is potentially active in these cells. Both IKKε and TBK1, but not their kinase mutant forms, activated the 3X-κB and IFN-β luciferase promoter constructs (Fig. 2, *A* and *B*). Unlike the 3X-κB promoter, the IFN-β promoter is considered a complex promoter regulated by coordinate actions of NF-κB and other transcription factors; therefore, it is not considered to be regulated exclusively by NF-κB. These results are similar to those of Shimada *et al.* (6). Interestingly, in all of the assays performed, TBK1 was observed to be a better activator of the reporters (Fig. 2, *A* and *B*). Analysis of the effects of different concentrations of IKKε on activation of the 3X-κB luciferase reporter showed that -fold induction of luciferase activity is proportional to the IKKε plasmid concentration, whereas there was no significant induction with the vector control or with the kinase mutant (KM) form of IKKε (Fig. 2*C*).

IKKε Expression Induces NF-κB DNA Binding Activity—We next investigated whether IKKε expression can induce NF-κB binding to a consensus DNA target sequence. FLAG-tagged IKKε was transiently expressed in HEK 293T cells for ~48 h, and nuclear extracts were prepared for EMSAs. As shown in

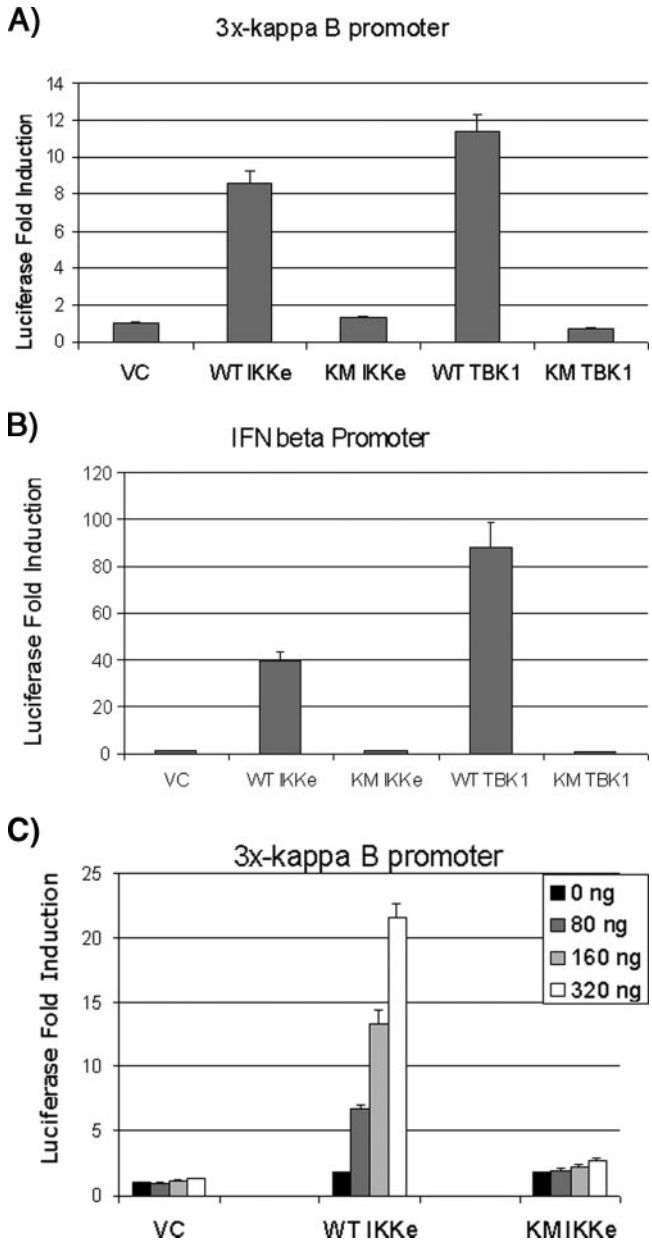


FIGURE 2. **IKKε and TBK1 but not their kinase mutant forms activate NF-κB-dependent reporter and IFNβ promoter.** HEK 293T cells seeded in 24-well plate were transiently co-transfected the next day with an expression vector for IKKε, TBK1, or their kinase mutant forms and vector encoding a reporter gene for 3x-κB reporter (*A*) and IFN-β promoter (*B*). Experiments were done at least twice in triplicate, and luciferase reporter gene activity was measured 24–48 h after transfection. *C*, IKKε activates NF-κB-dependent promoter in a concentration-dependent manner. The indicated concentration of IKKε and its kinase mutant form were co-transfected with 3x-κB reporter gene constructs in a 48-well plate.

Fig. 3*A*, IKKε effectively induced the DNA binding activity of NF-κB. TNF-α is included in this experiment for comparison purposes. It should be noted that there is basal NF-κB DNA binding activity in the VC lane (*lane 1*), which is better visualized given longer exposure times. TNF-α stimulation of IKKε-overexpressing cells led to more DNA binding activity, but this increase appears to be an additive effect of TNF-α and IKKε rather than a synergistic effect. Western blot analysis of cytoplasmic extracts, shown in the *lower panel*, demonstrates expression levels of IKKε. We next aimed to investigate the

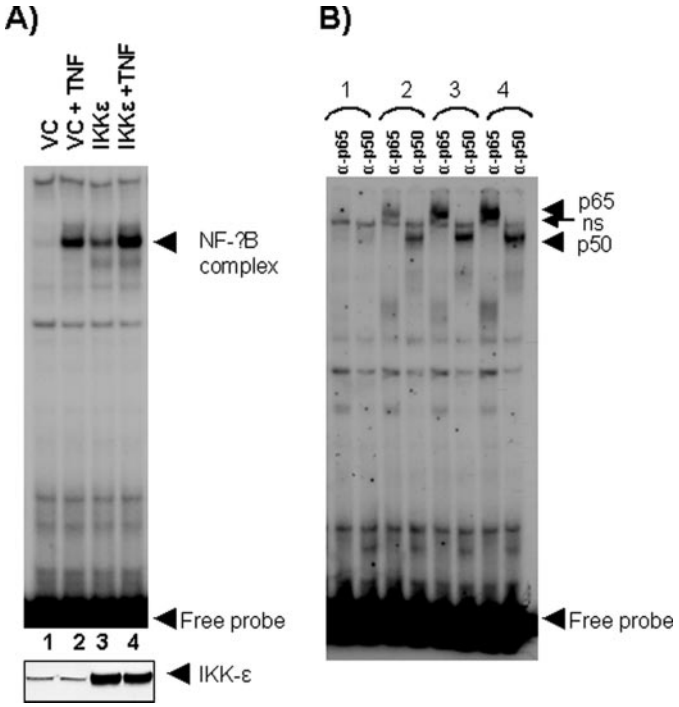


FIGURE 3. **IKKε induces significant NF-κB DNA binding activity.** *A*, HEK 293T cells seeded in 10-cm dishes were transfected with expression vector for IKKε or empty vectors for 48 h. The indicated cells were treated with 10 ng/ml TNF-α for 30 min. Protein-DNA complex was resolved by EMSA technique as described under "Materials and Methods." TNF-α is included in the experiment for control purposes. The NF-κB complex and the free probe are indicated by the *arrows*. Western blot analysis of cytoplasmic extracts is shown in the *lower panel* for the analysis of IKKε expression. *B*, NF-κB complex induced by IKKε and TNFα is mainly composed of p65 and p50 heterodimers. Each *lane* in *A* has been shifted with the indicated antibodies. The *numbers* at the *top* (*1–4*) show the lane numbers in *A*. RelA/p65 or p50 binding activity was assessed by incubation of 5 μg of nuclear extracts with either p65- or p50-specific antibodies followed by EMSA. In the lanes where an NF-κB complex is detected (*2–4*), there is a supershifted band with the p65 and p50 antibodies. *ns*, nonspecific binding.

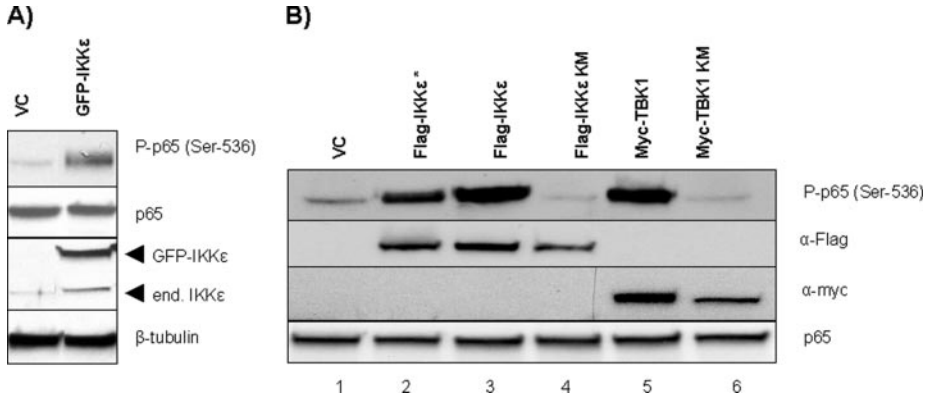


FIGURE 4. **IKKε and TBK1 expression leads to phosphorylation of endogenous p65 at Ser-536.** *A*, IKKε expression induces p65 phosphorylation and its own expression. HEK 293T cells were transiently transfected with 1 μg of the GFP-IKKε expression vector. Whole cell lysates were prepared 24 h after transfection and blotted with p65 phospho-specific antibody that detects phosphorylation at Ser-536 position (indicated as P-p65 (Ser-536)). Notably, endogenous IKKε expression is induced by ectopically expressed GFP-IKKε (indicated by *arrows*). The membrane was blotted with anti-p65 and anti-β-tubulin antibodies to demonstrate equal loading. *B*, WT IKKε and TBK1 but not their kinase mutant forms phosphorylate p65 at Ser-536 position. HEK 293T cells seeded in 6-well plate were transiently transfected with 1 μg of the WT FLAG-IKKε, KM FLAG-IKKε, WT myc-TBK1, and KM myc-TBK1 expression vectors. WT IKKε and TBK1 but not their kinase mutant forms induce p65 phosphorylation. Membranes were also blotted with anti-FLAG and anti-myc for IKKε and TBK1 expression. FLAG-IKKε* is WT IKKε with a mutation at 501 position (Thr-501 → Ala), which was thought to control IKKε activity; however, the mutation did not change its activity in terms of phosphorylating p65.

nature of the major NF-κB subunits in this bound complex by gel shift assay. For this purpose each of the reactions used in Fig. 3*A* was incubated with the indicated antibody and electrophoresed on a separate gel (Fig. 3*B*). The number at the *top* of the figure indicates the lane numbers (*lanes 1–4*) from the reactions used in Fig. 3*A*. In the lanes where an NF-κB complex was detected (*lanes 2–4*), there was a positive response with the p65 and p50 antibodies. Therefore, we concluded that the complex that bound to this consensus binding site is composed predominantly of p65/p50 heterodimers (Fig. 3*B*). It should be noted that a single nucleotide change can lead to binding of different NF-κB subunits (22, 23); therefore, we cannot exclude the possibility that other subunits might also be activated and bind to slightly different NF-κB binding sites. TBK1 expression effects on DNA binding activity of NF-κB yielded very similar results (data not shown).

IKKε and TBK1 Expression Leads to Phosphorylation of Endogenous p65 at Ser-536—Recent studies have shown that post-translational modification of NF-κB subunits, such as p65, contribute significantly to NF-κB transactivation potential (reviewed in Ref. 3). Phosphorylation of p65 at Ser-536 is proposed to be a key modification that potentiates p65 transactivation function, hence NF-κB activation (20, 24). We next tested if IKKε and TBK1 affect p65 phosphorylation. We tested whether ectopically expressed IKKε leads to phosphorylation of endogenous p65 at Ser-536. Expression of GFP-IKKε leads to a significantly higher level of p65 phosphorylation at the Ser-536 position (indicated as P-p65 (Ser-536)) (Fig. 4*A*). Utilization of GFP-tagged IKKε expression vector allows for distinguishing between ectopically expressed IKKε (GFP-IKKε) and endogenous IKKε. This experiment reveals that IKKε induces higher levels of endogenous IKKε (Fig. 4*A*), which has been proposed to be regulated by NF-κB (25). This observation suggests that ectopically expressed IKKε is able not only to induce phosphorylation of endogenous p65 but also to induce NF-κB-dependent gene expression. Next we examined if TBK1, an IKKε homolog, will also induce Ser-536 phosphorylation of p65. As shown in Fig. 4*B*, WT forms of both IKKε and TBK1 induced p65 Ser-536 phosphorylation; however, their kinase mutant forms (Lys-38 → Ala) did not lead to phosphorylation of p65. Indeed when analyzed in detail, kinase mutant forms appear to inhibit the basal level of endogenous Ser-536 phosphorylation (compare *lane 1* to *lanes 4* and *6*). It is important to note that the phospho-p65 Ser-536 antibody specifically detected only the phosphorylated form of p65 and did not cross-react with unphosphorylated p65 (see Fig. 4*B*).

Analysis of Inducible p65 Ser-536 Phosphorylation in IKKε-deficient Cells—Observing that exogenous IKKε induces p65 phosphorylation, we hypothesized that this post-

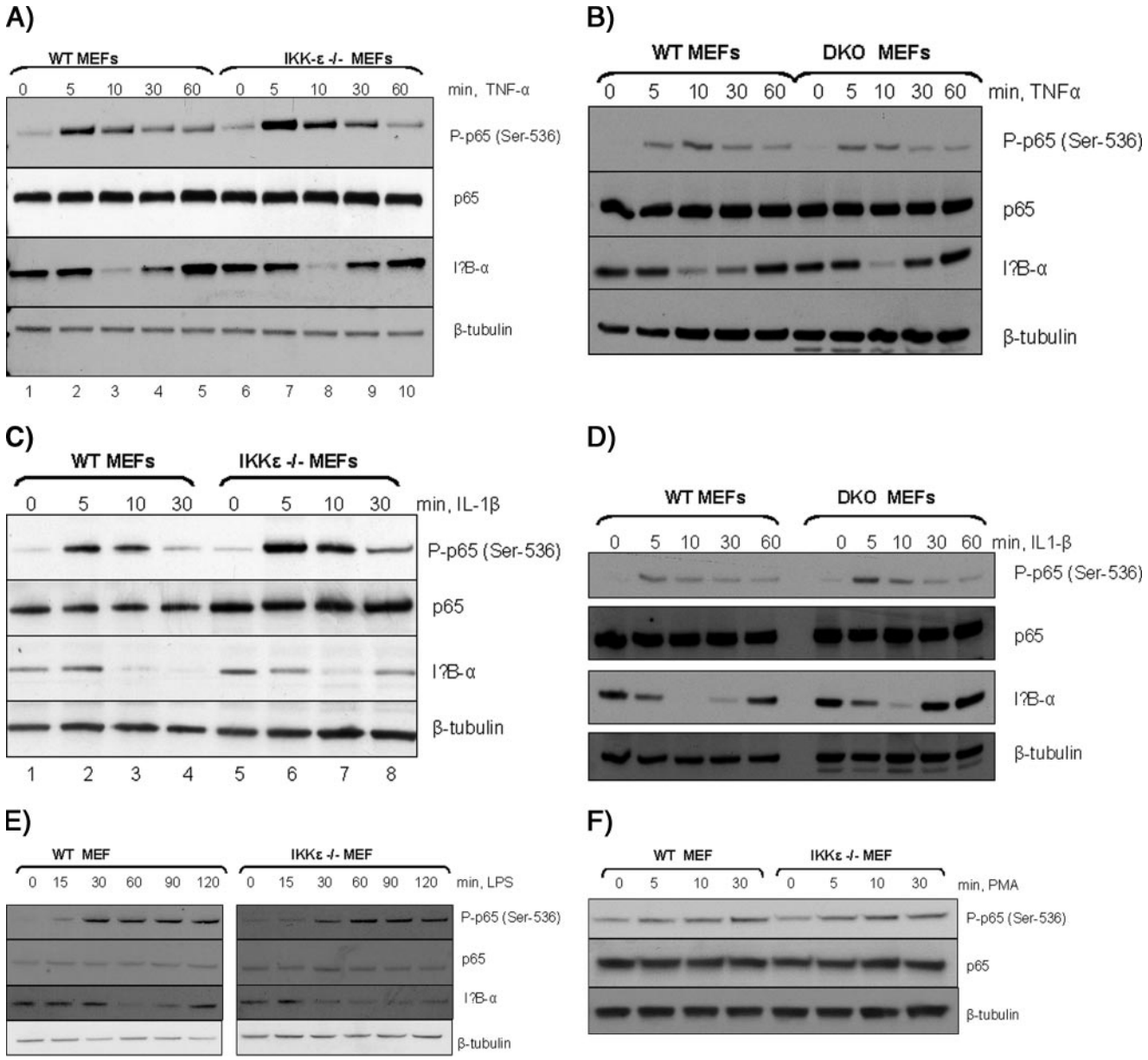


FIGURE 5. **p65 phosphorylation at Ser-536 position is normal in IKKε-deficient MEFs in response to different stimuli.** Cytokine induced p65 phosphorylation in IKKε singly deficient and IKKε and TBK1 doubly deficient MEFs is comparable to WT MEFs. Indicated MEF cells were seeded in 6-well plates, and at 70–80% confluency they were stimulated with 10 ng/ml TNFα (*A* and *B*), 10 ng/ml IL-1β (*C* and *D*), 1 μg/ml LPS (*E*), and 100 ng/ml PMA (*F*) for indicated time points. Whole cell extracts were prepared and blotted with indicated antibodies. Interestingly, in all of the stimuli tested, there was no significant difference in terms of either p65 phosphorylation pattern or IκBα degradation kinetic in IKKε-deficient or DKO MEFs compared with WT MEF cells. Analysis of β-tubulin levels confirmed that loading was essentially equivalent in all lanes.

translational modification on p65 might be defective in IKKε-deficient MEFs in response to NF-κB inducers. To test our hypothesis, IKKε-deficient and IKKε and TBK1 doubly deficient MEFs (DKO) were stimulated with TNFα, a well known NF-κB inducer, and compared with similarly treated WT MEFs. As seen in Fig. 5*A*, p65 was phosphorylated at the Ser-536 position in response to TNF-α as early as 5-min post-stimulation. Interestingly, IKKε-deficient cells showed essentially the same pattern of phosphorylation kinetics in response to TNFα stimulation. In addition to p65 phosphorylation, IκBα degradation was also normal in IKKε-deficient cells compared with WT MEF cells. Analysis of β-tubulin levels confirmed that loading was essentially equivalent in all lanes (Fig. 5*A*). To determine if the loss of TBK1 together with IKKε would effect the phosphorylation of p65 and IκBα degradation, WT MEFs and MEFs deficient for both IKKε and TBK1 (double knock-out MEFs (DKO)) have been used under similar experimental conditions. Both p65 phosphorylation and IκBα degradation were normal in DKO MEFs (Fig. 5*B*). This result indicated that NF-κB activation, as measured by p65 phosphorylation and IκBα degradation in response to TNF-α, is independent of IKKε and TBK1.

We next tested the effect of IL-1β on p65 phosphorylation as well as IκB degradation (Fig. 5*C*). IL-1β is, like TNF-α, a well known inducer of NF-κB. In as early as 5 min, p65 was phosphorylated maximally, however, maximal IκBα degradation

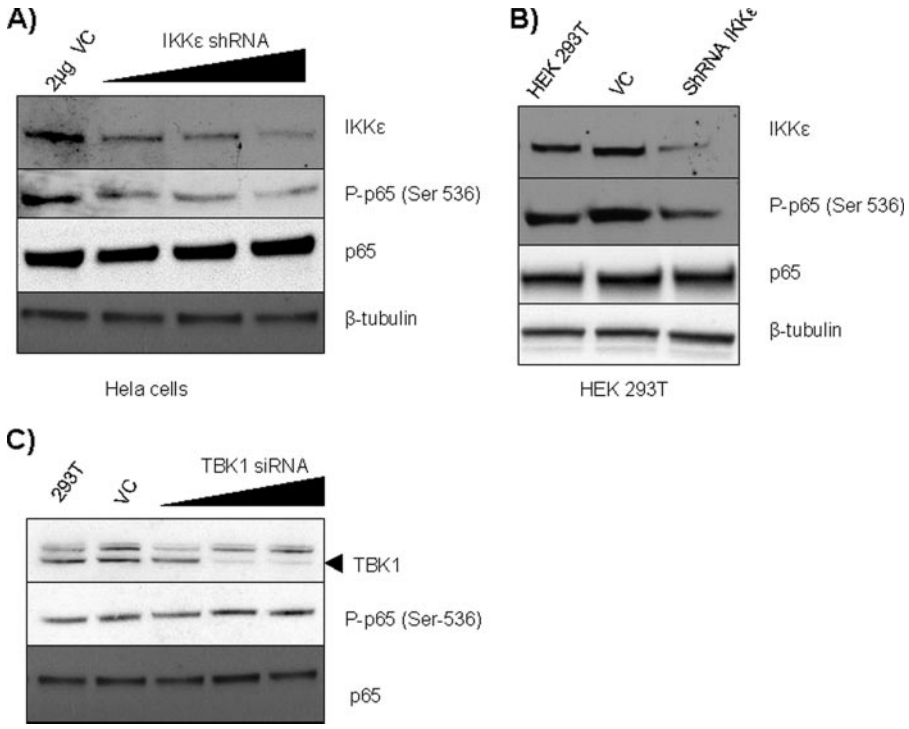


FIGURE 6. **Sustained knockdown of IKKε leads to impaired Ser-536 phosphorylation of p56.** *A* and *B*, vector encoding shRNA targeting IKKε or vector control (*VC*) alone was transfected in HeLa cells (*A*) in 6-well plate with increasing concentration of IKKε shRNA vector (1, 2, and 3 μg/well shRNA plasmid) or in HEK 293T cells (*B*) for 72 h. Lysate preparation and Western blots were done as described above. A significant knockdown in IKKε level was well correlated with reduction in p65 Ser-536 phosphorylation, whereas there is no change in total p65 level. β-tubulin level is shown to demonstrate equal loading in all lanes. *C*, siRNA targeting TBK1 was transfected in HeLa cells for 72 h. As shown, despite the significant reduction in TBK1 level, No significant change in p65 phosphorylation was observed.

was observed in 10 min. It is interesting again to observe that both p65 phosphorylation at Ser-536 and IκBα degradation were normal in IKKε-deficient cells compared with WT cells. In parallel studies, experiments were also performed in cells where both IKKε and TBK1 were deleted (DKO), and we observed no significant difference from WT cells relative to Ser-536 p65 phosphorylation or IκBα degradation after stimulation with TNF-α or IL-1β (Fig. 5*D*). These results suggest a minimal role of IKKε and TBK1 in cytokine-induced p65 phosphorylation and IκBα degradation.

The pathway to NF-κB activation in response to LPS has been characterized in molecular detail resulting in the discovery of a novel family of adapter proteins, which serve to regulate and polish up toll-like receptor responses. The first identified member of this adapter family was MyD88 (26). The importance of MyD88 in toll-like receptor signaling has been confirmed by the inability of MyD88-deficient mice to respond properly to a variety of toll-like receptor ligands, namely LPS, peptidoglycan, and bacterial CpG motifs (27, 28). Interestingly, analysis of MyD88-deficient cells in response to LPS demonstrated the existence of MyD88-independent, late NF-κB activation, and the induction of IRF-3-dependent genes, which has recently been verified to be regulated by IKKε and TBK1 kinases (11, 13, 15). In our experimental system, LPS stimulation of WT MEFs and IKKε-deficient cells showed similar levels of inducible p65 phosphorylation (Fig. 5*E*). As expected, the kinetics of the phosphorylation upon LPS stimulation is not as fast as TNF-α and IL-1β, but the pattern of phosphorylation is similar between WT and IKKε-deficient cells. In addition to p65 phosphorylation, IκBα degradation was also analyzed, and there was no defect in this process. We next tested the effect of PMA stimulation on p65 phosphorylation and did not observe any difference between WT and IKKε-deficient MEFs (Fig. 5*F*). Overall our data indicate that inducible Ser-536 phosphorylation is unaffected in IKKε-deficient cells and confirm that IKKε is not significantly involved in mechanisms associated with cytokine-, LPS-, or PMA-induced IκBα degradation.

IKKε Controls Constitutive p65 Ser-536 Phosphorylation—It was surprising to observe that IKKε and TBK1 expression led to the phosphorylation of endogenous p65 but that MEFs deficient for these kinases depict normal phosphorylation patterns. Because we had observed a correlation between IKKε and Ser-536 in certain cancer cells, we therefore hypothesized that these kinases might be involved in basal or constitutive p65 phosphorylation. Because the basal or constitutive level of p65 phosphorylation is quite low in MEF cells, the potential that IKKε could contribute to basal/constitutive levels of Ser-536 phosphorylation was investigated in HeLa and HEK 293T cells. These cells have higher levels of IKKε expression, constitutive p65 phosphorylation, and NF-κB activation compared with MEFs. To knock down IKKε, both plasmid-based shRNA and normal siRNA technologies have been utilized against IKKε mRNA. Additionally, an identical control plasmid, which contains a scrambled sequence with no homology to any known human gene product, has been utilized. Extracts from control-treated and siRNA-treated HeLa cells were analyzed for IKKε knockdown as well as for endogenous p65 Ser-536 phosphorylation. Our results indicated that transfection of shRNA against IKKε leads to sustained effective knockdown of IKKε. Furthermore, the reduction in IKKε protein level is well correlated with significant reduction in the basal level of Ser-536 phosphorylation in HeLa cells as compared with vector control-treated cells (Fig. 6*A*). Quantitative real-time-PCR analysis showed more than a 70% reduction in *ikkε* mRNA in HeLa cells when transfected with 2 μg of shRNA plasmid (data not shown). To show that this is not a cell line-specific observation, similar experiments have been performed in HEK 293T cells. In cells where the shRNA plasmid was transfected, IKKε level were significantly reduced. Again, a significant reduction in Ser-536 phosphorylation of p65 was observed, whereas vector control-treated cells exhibited no change in IKKε levels or Ser-536 phosphorylation (Fig. 6*B*). These results demonstrate that IKKε has a significant role in controlling the basal/consti-

tutive p65 phosphorylation at Ser-536 position in two cell lines. Interestingly, knockdown of TBK1 with siRNA did not show a significant change in p65 phosphorylation at Ser-536 position (Fig. 6*C*). This result indicates the differential role of IKKε and TBK1 in terms of controlling basal/constitutive p65 phosphorylation, at least in the cell types analyzed.

IKKε Knockdown in HeLa Cells Results in Reduced Constitutive Activity of an NF-κB-dependent Promoter—To better elucidate the role of IKKε in controlling NF-κB activity, and to determine if reduction in p65 phosphorylation upon IKKε knockdown is correlated with reduced constitutive NF-κB activity, we have analyzed NF-κB-dependent luciferase reporter assays in HeLa cells. These cells were transfected with either negative control siRNA and or with IKKε siRNA for 48 h followed by transfection with the 3x-κB luciferase promoter construct. Importantly, knockdown of IKKε in a dose-dependent manner resulted in reduced promoter activity as compared with the control construct (Fig. 7). This result indicates that IKKε controls a significant portion of NF-κB-dependent activity in HeLa cells, presumably through its ability to control Ser-536 p65 phosphorylation.

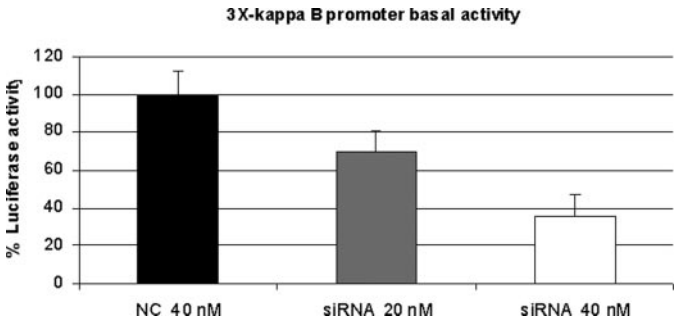


FIGURE 7. **IKKε knockdown in HeLa cells leads to reduction in basal activity of NF-κB dependent promoter.** HeLa cells seeded in 6-well plates were transfected by siRNA targeting IKKε or negative control (*NC*) siRNA at the indicated concentrations for 48 h. Then cells were split into 24-well plates and were transfected with 3X-κB promoter and *Renilla* luciferase promoter as an internal control. siRNA was also included in this transfection complex to give a second hit for IKKε knockdown. At indicated concentrations of siRNA (20 and 40 nM), knockdown efficiency of IKKε mRNA was 58 and 79%, respectively, as measured by quantitative real-time-PCR (data not shown). Reduction in IKKε level is well correlated with the reduction in basal activity of an NF-κB-dependent promoter.

IKKε and p65 Phosphorylation Positively Mediate HeLa Cell Proliferation—After observing that IKKε controls basal p65 phosphorylation and NF-κB activity in certain cancer cells, we asked if IKKε provides cell growth/survival functions. For this purpose, MTT cell proliferation assays were performed in HeLa cells transfected with shRNA against IKKε or with a non-phosphorylatable form of p65 (S536A) expression construct. For the data presented in Fig. 8 (*A* and *B*), HeLa cells were seeded in 6-well plates and were transfected the next day with the indicated plasmids. 24 h after transfection, cells were reseeded on 96-well plates, and the MTT proliferation assay was performed 24 and 48 h later. When IKKε was knocked down by shRNA, a significant reduction in cell proliferation was observed at the 48-h time point as compared with control cells transfected with the scrambled shRNA plasmid (Fig. 8*A*). In a similar experimental setting, we tested the effect of IKKε-mediated p65 phosphorylation on cell proliferation. For the purpose, IKKε was transfected with WT or a p65 mutant (S536A) that cannot be phosphorylated at the Ser-536 position. MTT assays read after 24 and 48 h shows that cells with mutant p65 do not proliferate efficiently when compared with cells expressing WT p65. This experiment indicates that p65 phosphorylation at Ser-536 is important for HeLa cell proliferation.

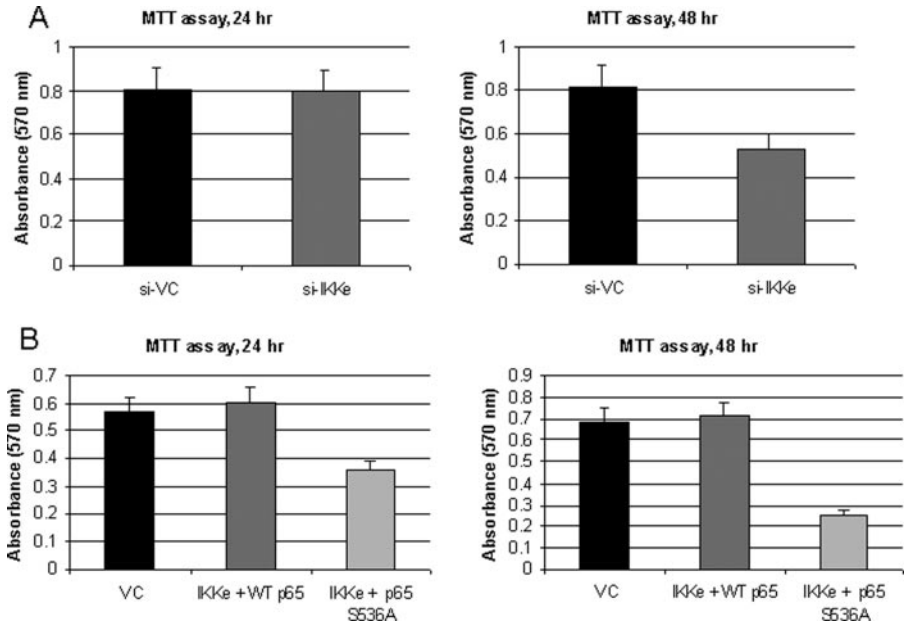


FIGURE 8. **IKKε and p65 phosphorylation mediate cell proliferation.** HeLa cells seeded in 6-well plates were transfected with the indicated plasmids for 24 h, and then cells were trypsinized and seeded in 96-well plates in triplicates. After the indicated time points, an MTT assay was performed. Results are representative of two independent experiments done in triplicates. When the IKKε level was modified by shRNA, cell proliferation was not as efficient as control shRNA-transfected cells. IKKε knockdown with shRNA after 48 h reduced cell proliferation significantly (*A*). *Si-VC* and *Si-IKKε* represent vector control shRNA and shRNA against IKKε, respectively. IKKε was also overexpressed together with WT and S536A mutant version of p65 to test the significance of IKKε-mediated p65 phosphorylation (*B*). Although IKKε- and WT p65-transfected cells proliferated slightly better than vector control-transfected cells, when IKKε was overexpressed with the S536A mutant version of p65, which cannot be phosphorylated at the Ser-536 position, there was significantly less cell proliferation. These data suggest that both IKKε level and p65 phosphorylation status are important for proper cell proliferation.

DISCUSSION

The majority of studies analyzing NF-κB activation have focused on induction of this transcription factor downstream of cytokine or LPS-dependent signaling. This response is generally dependent on the classic IKK complex, containing IKKα and IKKβ. Additional evidence has indicated that, besides the nuclear translocation of NF-κB, post-translational modifications, like p65 phosphorylation, are required to efficiently activate NF-κB-dependent gene transcription (20, 29–34). It is also well established that a number

of cells, particularly those of cancerous origin, exhibit significantly elevated levels of basal or constitutive NF-κB activity. In many cases, the origins of this activity remain unclear.

Here we show that several cancer cell lines, along with the SV40 large T-immortalized 293 cell line, exhibit relatively high levels of expression of IKKε. This is interesting, because IKKε is normally considered a kinase that is induced quantitatively by LPS or cytokines. We have investigated a potential role for IKKε and TBK1, kinases homologous to the catalytically active IKKα and IKKβ subunits, in controlling NF-κB activity, with the focus being phosphorylation of p65 at the Ser-536 position. Experiments were initiated to study IKKε- and TBK1-induced NF-κB-dependent promoter activation.

In agreement with previous results (10, 11), IKKε and TBK1, but not their kinase mutant forms, strongly activate NF-κB-regulated reporter constructs. It is important to note that, unlike the 3X-κB promoter, the IFN-β promoter is a complex promoter regulated by coordinate actions of NF-κB and other transcription factors, therefore it is not considered to be regulated exclusively by NF-κB. To confirm our reporter assays, gel shift assays have been performed. As expected, IKKε and TBK1 induced significant NF-κB DNA binding activity. Supershift assays identified p65 and p50 as main subunits of NF-κB complex. Recent studies have shown that post-translational modification of NF-κB subunits, such as p65 phosphorylation, contribute significantly to NF-κB activation. Phosphorylation of p65 at Ser-536 is proposed to be a key modification that potentiates p65 transactivation function, and hence NF-κB activation ability (20, 24, 32).

Recently, it has been reported that overexpression of IKKε or TBK1 together with p65, leads to the phosphorylation of ectopically expressed p65 at Ser-536 (19), however, this group has not analyzed the endogenous phosphorylation at Ser-536 of p65. Our results clearly support the hypothesis that the kinase activity of IKKε and TBK1 may significantly contribute to the constitutive level of S536 phosphorylation of p65. We have also observed that IKKε induces its own expression, which has been shown to be regulated by NF-κB. These data indicate that ectopically expressed IKKε induces p65 phosphorylation, NF-κB activation, and NF-κB-dependent gene expression. It also raises the possibility that IKKε functions in an autoregulatory loop, leading to its own expression.

It was interesting to observe that IKKε-deficient cells show a normal pattern of cytokine-inducible phosphorylation of p65 and IκBα degradation when compared with WT MEFs. We have tested a series of well known NF-κB inducers (IL-1β, LPS, and PMA) that are known to activate NF-κB by utilizing different signaling pathways. Compared with WT MEFs, IKKε-null cells (and DKO MEFs for both IKKε and TBK1) allowed inducible RelA/p65 phosphorylation to the same extent. Why is there no defect in inducible Ser-536 phosphorylation of p65 in IKKε-deficient cells? The first plausible explanation to this question is that the classic IKK signalsome complex is still intact in IKKε-deficient cells. Therefore, this complex likely compensates for the loss of IKKε. Secondly, there are other known and unknown kinases, in addition to IKK complex, that have been claimed to mediate Ser-536 phosphorylation of p65 (20, 34, 35), and in the same way, they may still induce phosphorylation in IKKε-deficient cells. The third explanation to this question is that IKKε and TBK1 are not involved in cytokine-induced p65 phosphorylation, but rather they are involved in the basal/constitutive level of p65 phosphorylation. In one set of experiments, this possibility has been investigated. Because MEF cells have low levels of basal p65 phosphorylation and low levels of IKKε, the potential that IKKε and TBK1 might be involved in constitutive p65 phosphorylation has been investigated in HeLa and HEK 293T cells that demonstrate higher levels of IKKε and constitutive p65 phosphorylation at Ser-536 position. IKKε was knocked down by plasmid-based shRNA technology in both HeLa cells and HEK 293T cells. Importantly, shRNA transfection leads to sustained knockdown of IKKε, and more importantly, reduction in the IKKε level is well correlated with significant reduction in the basal level of Ser-536 phosphorylation (Fig. 6), whereas in vector control-transfected cells there is no change in IKKε level and Ser-536 phosphorylation of p65. Interestingly knockdown of TBK1 did not reduce the basal level of p65 phosphorylation. Surprisingly these data suggest that TBK1 and IKKε are not entirely orthologues at least in controlling basal phosphorylation of p65. As suggested earlier (15), IKKε and TBK1 might not be redundant in every signaling pathway that they affect. Thus the data presented here clearly show that IKKε does not mediate cytokine-induced p65 phosphorylation at the Ser-536 position but has a significant role in basal and constitutive phosphorylation of p65 at least in certain cancer cells and in 293T cells. Basal p65 phosphorylation is well correlated with constitutive NF-κB activity, which has been implicated in the pathogenesis of many diseases, including cancer. The first evidence to our conclusion that IKKε mediates constitutive NF-κB activity came from a recent study published while this report was in preparation (36). In that study, Eddy *et al.*, provided evidences that IKKε contributes to the pathogenesis of breast cancer. Expression of a kinase-inactive form of IKKε blocked breast cancer cell colony formation. The results presented in that study are consistent with the findings presented here. We have shown that IKKε contributes to the basal/constitutive p65 phosphorylation and NF-κB activity as measured by EMSA and NF-κB-driven luciferase promoter activity. Furthermore, we have also shown that knockdown of IKKε or overexpression of mutated version of p65 (S536A) negatively effects the cell proliferation. These findings indicate an important role of IKKε and p65 phosphorylation in cancer cell proliferation.

There have been reports suggesting that IKKε and TBK1 may function as IκB kinase kinases (8, 37). Therefore they might function upstream of the classic IKK complex (IKKα/β/γ). So we questioned if the phosphorylation of p65 at Ser-536 is a direct or indirect effect of IKKε/TBK1. In other words, IKKε and TBK1 might have activated classic IKKs, which then lead to the phosphorylation of p65. To test this, we tried to detect activation of IKKβ and IKKα by probing the same blots in Fig. 4 with commercially available, phospho-IKKα/β antibodies; however, we could not detect any phosphorylation (data not shown). Although we cannot rule out that IKKε or TBK1 might function as IκB kinase kinases, our data support a model where these two kinases are direct effectors of p65 activation. There is evidence in the literature supporting this model. First of all, it

has been clearly shown that stimulus-coupled IκB degradation and p65 nuclear translocation and DNA binding activity of NF-κB is normal in IKKε and TBK1-deficient cells despite the fact that there is impaired NF-κB-dependent gene transcription (15, 16, 38). This evidence supports the fact that the activity of IKKα and IKKβ is normal in IKKε- or TBK1-deficient cells, because there is normal IκB degradation, normal p65 nuclear translocation, and normal DNA binding activity. We believe that our data, together with these findings, emphasize the RelA/p65 as the physiological target of IKKε and TBK1 under basal growth situations, at least in certain cells. If IKKε and TBK1 were upstream of classic IKKs, one would expect a deficiency in one of the above processes which are tightly regulated by IKKα and -β.

Very recently an article has been published indicating that IKKε mediates inducible phosphorylation of NF-κB p65 at serine 468 but not at serine 536 during T-cell co-stimulation (39). However, the outcome of the phosphorylation at Ser-468 by IKKε has not been shown, and the phosphorylation at this site by glycogen synthase kinase-3β has been claimed by the same group to be associated with negative NF-κB regulation (40).

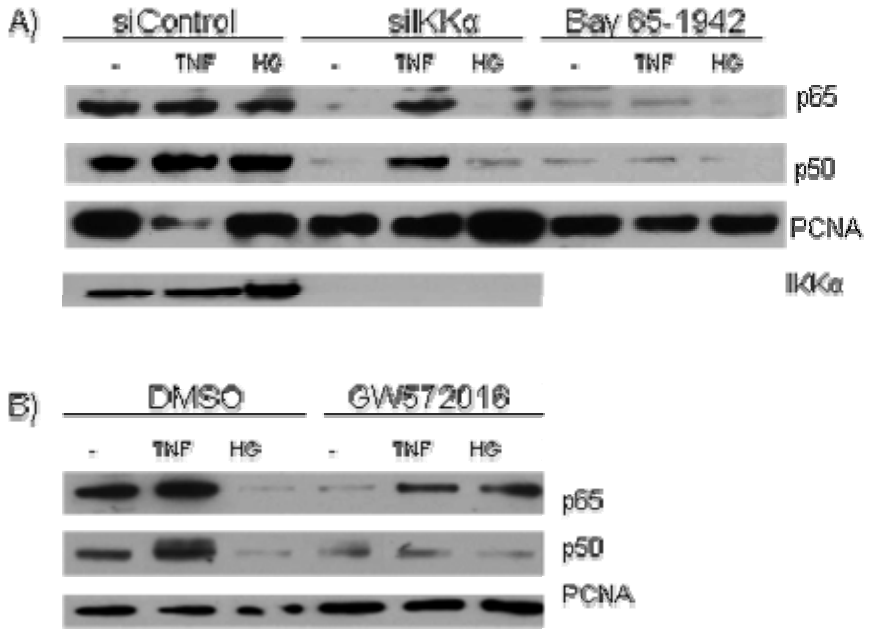
To summarize, we have provided evidence that IKKε, and not TBK1, controls the constitutive NF-κB activity in certain cancer cells and in 293T cells. This evidence is supported by siRNA experiments and by associated reporter studies. It is presently unclear whether IKKε functions separately from the classic IKK complex, or through distinct regulatory pathways. It is also unclear whether IKKε is the kinase that directly controls Ser-536 p65 phosphorylation. Nevertheless, the data indicate a potentially important role for IKKε in controlling at least part of the constitutive NF-κB activity generated in certain cancer cells, with subsequent downstream effects on cancer cell proliferation.

Acknowledgments—We gratefully acknowledge Dr. John Hiscott (McGill University) for providing the TBK1 and the KM TBK1 (K38A) vectors; Dr. Tom Maniatis (Harvard University) for providing the IFNβ luciferase construct; and Dr. Shizuo Akira (Osaka University) for providing the IKKε and IKKε/TBK1 double null fibroblasts. We also acknowledge Dr. Raquel Sitcheran and Dr. Noel Hawke for carefully and critically reading the manuscript.

REFERENCES

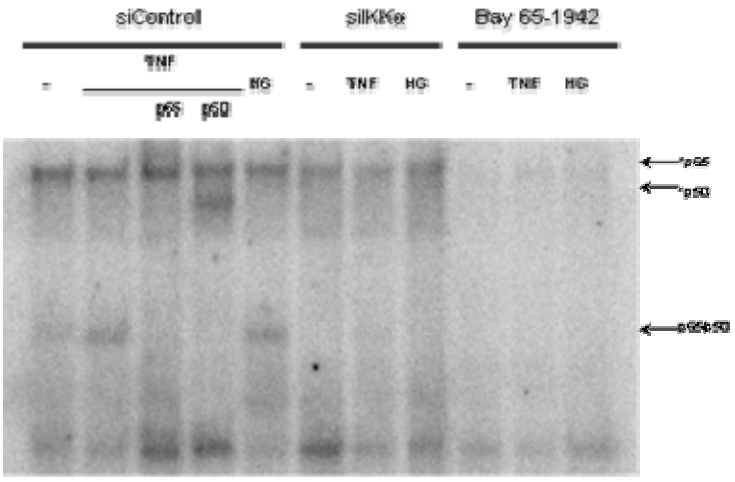
1. Hayden, M. S., and Ghosh, S. (2004) *Genes Dev.* **18,** 2195–2224
2. Israel, A. (2000) *Trends Cell Biol.* **10,** 129–133
3. Ghosh, S., and Karin, M. (2002) *Cell* **109,** (suppl.), S81–S96
4. Peters, R. T., and Maniatis, T. (2001) *Biochim. Biophys. Acta* **1471,** M57–M62
5. Peters, R. T., Liao, S. M., and Maniatis, T. (2000) *Mol. Cell.* **5,** 513–522
6. Shimada, T., Kawai, T., Takeda, K., Matsumoto, M., Inoue, J., Tatsumi, Y., Kanamaru, A., and Akira, S. (1999) *Int. Immunol.* **11,** 1357–1362
7. Pomerantz, J. L., and Baltimore, D. (1999) *EMBO J.* **18,** 6694–6704
8. Tojima, Y., Fujimoto, A., Delhase, M., Chen, Y., Hatakeyama, S., Nakayama, K., Kaneko, Y., Nimura, Y., Motoyama, N., Ikeda, K., Karin, M., and Nakanishi, M. (2000) *Nature* **404,** 778–782
9. Kishore, N., Huynh, Q. K., Mathialagan, S., Hall, T., Rouw, S., Creely, D., Lange, G., Caroll, J., Reitz, B., Donnelly, A., Boddupalli, H., Combs, R. G., Kretzmer, K., and Tripp, C. S. (2002) *J. Biol. Chem.* **277,** 13840–13847
10. Fitzgerald, K. A., McWhirter, S. M., Faia, K. L., Rowe, D. C., Latz, E., Golenbock, D. T., Coyle, A. J., Liao, S. M., and Maniatis, T. (2003) *Nat. Immunol.* **4,** 491–496
11. Sharma, S., tenOever, B. R., Grandvaux, N., Zhou, G. P., Lin, R., and Hiscott, J. (2003) *Science* **300,** 1148–1151
12. Bonnard, M., Mirtsos, C., Suzuki, S., Graham, K., Huang, J., Ng, M., Itie, A., Wakeham, A., Shahinian, A., Henzel, W. J., Elia, A. J., Shillinglaw, W., Mak, T. W., Cao, Z., and Yeh, W. C. (2000) *EMBO J.* **19,** 4976–4985
13. McWhirter, S. M., Fitzgerald, K. A., Rosains, J., Rowe, D. C., Golenbock, D. T., and Maniatis, T. (2004) *Proc. Natl. Acad. Sci. U. S. A.* **101,** 233–238
14. Takeuchi, O., Hemmi, H., and Akira, S. (2004) *J. Endotoxin. Res.* **10,** 252–256
15. Hemmi, H., Takeuchi, O., Sato, S., Yamamoto, M., Kaisho, T., Sanjo, H., Kawai, T., Hoshino, K., Takeda, K., and Akira, S. (2004) *J. Exp. Med.* **199,** 1641–1650
16. Perry, A. K., Chow, E. K., Goodnough, J. B., Yeh, W. C., and Cheng, G. (2004) *J. Exp. Med.* **199,** 1651–1658
17. Sankar, S., Chan, H., Romanow, W. J., Li, J., and Bates, R. J. (2005) *Cell Signal.* **18,** 982–993
18. Nomura, F., Kawai, T., Nakanishi, K., and Akira, S. (2000) *Genes Cells* **5,** 191–202
19. Buss, H., Dorrie, A., Schmitz, M. L., Hoffmann, E., Resch, K., and Kracht, M. (2004) *J. Biol. Chem.* **279,** 55633–55643
20. Sizemore, N., Leung, S., and Stark, G. R. (1999) *Mol. Cell. Biol.* **19,** 4798–4805
21. Mayo, M. W., Norris, J. L., and Baldwin, A. S. (2001) *Methods Enzymol.* **333,** 73–87
22. Leung, T. H., Hoffmann, A., and Baltimore, D. (2004) *Cell* **118,** 453–464
23. Natoli, G. (2006) *FEBS Lett.* **580,** 2843–2849
24. Fujita, F., Taniguchi, Y., Kato, T., Narita, Y., Furuya, A., Ogawa, T., Sakurai, H., Joh, T., Itoh, M., Delhase, M., Karin, M., and Nakanishi, M. (2003) *Mol. Cell. Biol.* **23,** 7780–7793
25. Wang, N., Ahmed, S., and Haqqi, T. M. (2005) *Gene* (*Amst.*) **353,** 118–133
26. Burns, K., Martinon, F., Esslinger, C., Pahl, H., Schneider, P., Bodmer, J. L., Di Marco, F., French, L., and Tschopp, J. (1998) *J. Biol. Chem.* **273,** 12203–12209
27. Adachi, O., Kawai, T., Takeda, K., Matsumoto, M., Tsutsui, H., Sakagami, M., Nakanishi, K., and Akira, S. (1998) *Immunity* **9,** 143–150
28. Kawai, T., Adachi, O., Ogawa, T., Takeda, K., and Akira, S. (1999) *Immunity* **11,** 115–122
29. Zhong, H., May, M. J., Jimi, E., and Ghosh, S. (2002) *Mol. Cell.* **9,** 625–636
30. Zhong, H., Voll, R. E., and Ghosh, S. (1998) *Mol. Cell.* **1,** 661–671
31. Wang, D., and Baldwin, A. S., Jr. (1998) *J. Biol. Chem.* **273,** 29411–29416
32. Sakurai, H., Chiba, H., Miyoshi, H., Sugita, T., and Toriumi, W. (1999) *J. Biol. Chem.* **274,** 30353–30356
33. Madrid, L. V., Mayo, M. W., Reuther, J. Y., and Baldwin, A. S., Jr. (2001) *J. Biol. Chem.* **276,** 18934–18940
34. Madrid, L. V., Wang, C. Y., Guttridge, D. C., Schottelius, A. J., Baldwin, A. S., Jr., and Mayo, M. W. (2000) *Mol. Cell. Biol.* **20,** 1626–1638
35. Doyle, S. L., Jefferies, C. A., and O'Neill, L. A. (2005) *J. Biol. Chem.* **280,** 23496–23501
36. Eddy, S. F., Guo, S., Demicco, E. G., Romieu-Mourez, R., Landesman-Bollag, E., Seldin, D. C., and Sonenshein, G. E. (2005) *Cancer Res.* **65,** 11375–11383
37. Campbell, K. J., and Perkins, N. D. (2004) *Biochem. Soc. Trans.* **32,** 1087–1089
38. Kravchenko, V. V., Mathison, J. C., Schwamborn, K., Mercurio, F., and Ulevitch, R. J. (2003) *J. Biol. Chem.* **278,** 26612–26619
39. Mattioli, I., Geng, H., Sebald, A., Hodel, M., Bucher, C., Kracht, M., and Schmitz, M. L. (2006) *J. Biol. Chem.* **281,** 6175–6183
40. Buss, H., Dorrie, A., Schmitz, M. L., Frank, R., Livingstone, M., Resch, K., and Kracht, M. (2004) *J. Biol. Chem.* **279,** 49571–49574

Fig. 1. A. IKKα and IKKβ control nuclear levels of p65 and p50 in Her-2/neu positive Breast cancer cells. B. Inhibition of Her-2/neu blocks constitutive levels of p65 and p50 in BT474 cells.



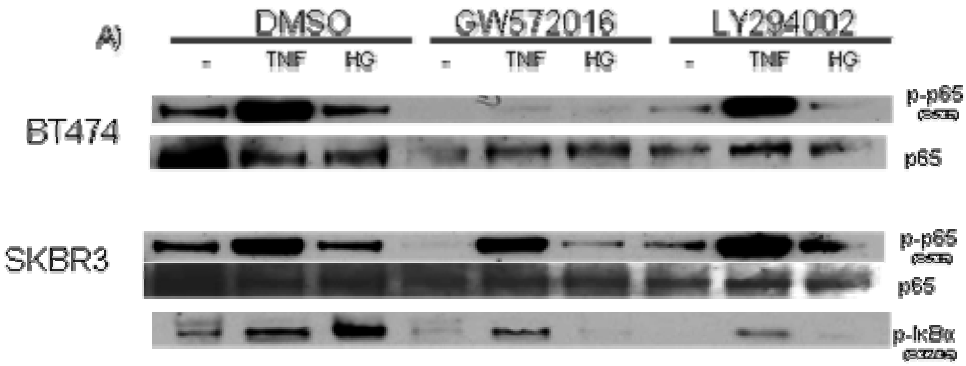
A) BT474 cells were treated with control or IKKα siRNA (100 nM) for three days or IKKβ inhibitor Bay 65-1942 (1 μM) for 2 hours, and stimulated with TNF (10 ng/ml) or Heregulin β (50 ng/ml) for 15 minutes and 2 hours respectively. Western blots of nuclear extracts were probed for NF-κB subunits p65 and p50.
B) BT474 cell lines were treated with pan-EGF R/Her2 inhibitor GW572016 (lapatinib) (1 μM) overnight and nuclear extracts were probed for NF-κB subunits.

Fig. 2. Results similar to those presented in figure 1, but using a different Her-2/neu positive cell line (SKBR3) and analyzed by electrophoretic mobility shift assay.



EMSA gel of SKBR3 nuclear extracts treated with control or IKKα siRNA or IKKβ inhibitor Bay 65-1942 as previously described. Cells were stimulated with TNFα or Heregulinβ as previously described. DNA binding measured using UV-21 NF-κB ^{32}P binding probe. Supershift bands labeled with asterisk, p65/p50 labeled with an arrow.

Fig. 3. Results similar to those presented in Fig. 1A. Immunoblotting for 65 in both cell lines with Her-2/neu inhibitor (GW572016) and PI3K inhibitor (LY294002).



BT474 and SKBR3 cells were treated with GW2016 (1 μM overnight) and LY294002 (10 μM 2 hours) and whole cell extracts were probed for phospho-p65 (Serine 536) and phospho-IκBα (Serine 32/36).